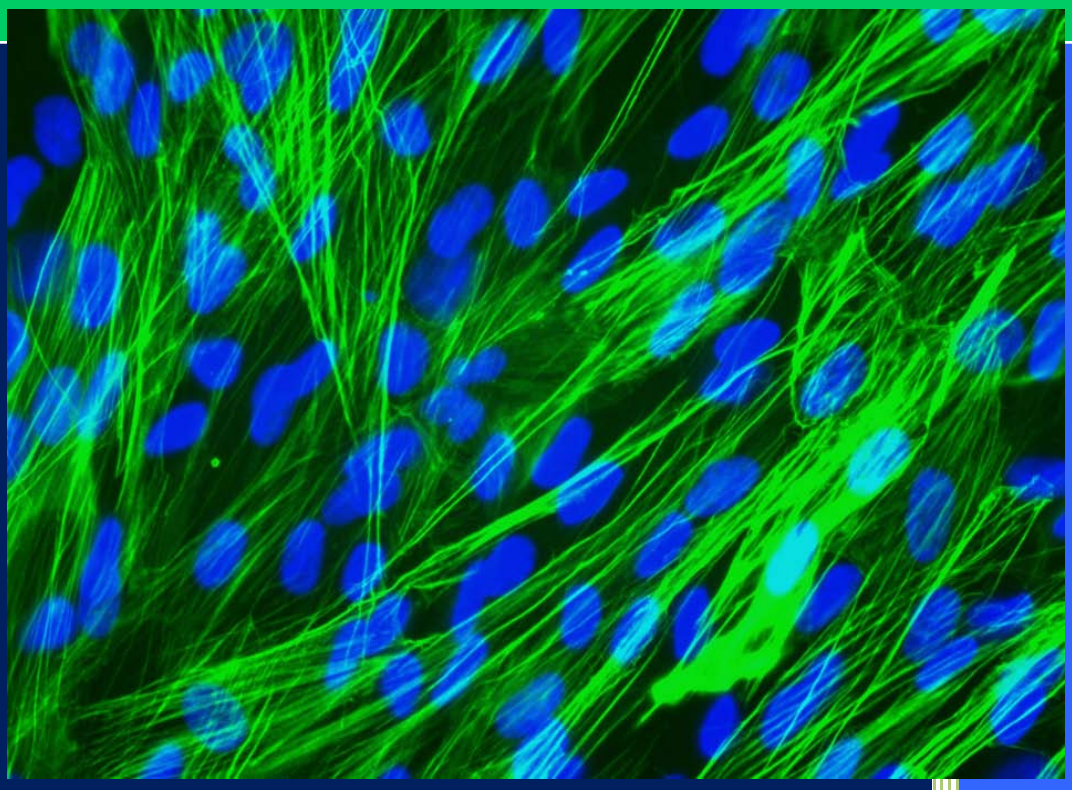
Doctoral Thesis

Effect of male sex hormones on neuronal function and vascular remodeling in the mesenteric artery



Lara del Campo Milán

Facultad de Medicina

Universidad Autónoma de Madrid

Madrid, 2012

Cover picture: Human pulmonary artery smooth muscle cells, alpha actin is immunostained in green and nuclei are stained in blue, 40X. With permission from http://www.sciencellonline.com/site/productInformation.php?keyword=3110 .

UNIVERSIDAD AUTÓNOMA DE MADRID

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Lara del Campo Milán

Madrid, 2012

Mercedes Ferrer Parra, Profesora Titular del Departamento de Fisiología de la Facultad de Medicina de la Universidad Autónoma de Madrid,

CERTIFICA:

Que Lara del Campo Milán ha realizado bajo su dirección el Trabajo **"Effect of male sex hormones on neuronal function and vascular remodeling in the mesenteric artery"** como Tesis Doctoral para optar al Grado de Doctor.

Madrid, 12 de Marzo de 2012

Fdo.: Dra. Mercedes Ferrer Parra

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“Los que aseguran que es imposible no deberían interrumpir a los que estamos intentándolo.”

Thomas Alva Edison

CONTENTS

ABSTRACT

The aim of this thesis was to investigate the role of endogenous male sex hormones on the neuroeffector mechanisms and vascular remodeling in the rat mesenteric artery, thus tackling the two mechanisms that control peripheral vascular resistances: vascular tone and vascular structure.

Previous studies on vascular tone have reported that orchidectomy does not modify the vasoconstrictor response induced by electrical field stimulation (EFS), although the response to exogenous noradrenaline (NA) is diminished, suggesting that EFS-induced NA release could be increased in arteries from orchidectomized rats. Besides, pre-junctional β-adrenoceptors can up-regulate the release of NA and neuronal nitric oxide (NO). Since female sex hormones have been reported to modulate the function of postsynaptic β-adrenoceptors in rat mesenteric arteries, we hypothesized that that male sex hormones could influence presynaptic β-adrenoceptor function, thereby affecting NA and neuronal NO release.

Modulation of the release and function of thromboxane A_2 (TXA_2) by male sex hormones has been previously described, although its role in the response to EFS had not been studied yet. Therefore we analyzed the effect of endogenous male sex hormones on the EFS-induced release of TXA_2 as well as its role in the vasoconstrictor response to EFS.

Vascular structure also determines vascular resistances. Sex hormones are known to influence the mechanisms controlling vascular structure, although their effect on the eutrophic inward remodeling has not been elucidated yet. Thus, the possible preventive effect of testosterone on inward remodeling induced by sustained vasoconstriction was tested in small mesenteric arteries from male rats. Since transglutaminase 2 (TG2) is involved in inward remodeling the role of this enzyme was also investigated.

For the studies on vascular tone, endothelium denuded mesenteric arteries from control and orchidectomized Sprague-Dawley rats were used. The effect of β-adrenoceptors activation and the role of TXA_2 in the vasoconstrictor response induced by EFS were analyzed. NA and neuronal NO release induced by EFS were measured, and the role of β-adrenoceptors and TXA_2 on the release of these neurotransmitters was analyzed. EFS-induced TXA_2 and prostaglandin I_2 (PGI_2) release were also measured on both groups of rats.

The results show that orchidectomy does not alter EFS-induced NA release, but it does increase EFS-induced TXA_2 release which would account for the maintenance of the EFS-induced vasoconstrictor response in spite of the decreased sensitivity to NA. β-adrenoceptor activation increases NO and NA release only in segments from orchidectomized rats confirming that male sex hormones influence β-adrenoceptor function. Orchidectomy also modifies the role of TXA_2 in the EFS-induced response, affecting at least two mechanisms, the NO release and response, and the PGI_2 release. In the control group, inhibition of TXA_2 formation decreases the EFS-response by increasing NO release and response, while in segments from orchidectomized rats, inhibition of TXA_2 formation does not alter the response to EFS by increasing the release of PGI_2 release, which mediates vasoconstriction.

For the studies on vascular remodeling, small mesenteric arteries from Wistar rats were used. Testosterone induced a dose-dependent vasodilation in arteries from male rats, which was inhibited by preincubation with the NO synthase inhibitor N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), indicating that testosterone induced NO release. When arteries were cannulated, pressurized, and kept in organ culture with endothelin-1 (ET-1) for three days we observed inward remodeling. This was significantly inhibited by testosterone and by the transglutaminase 2 (TG2) inhibitor L682.777. ET-1 did not significantly modify the TG2 activity and this was not modified by incubation with testosterone. It was concluded that testosterone prevent constriction-induced inward remodeling through mechanisms other that inactivation of TG2.

In summary, this thesis show that vascular function is under the influence of gonadal function. Orchidectomy modifies the neuronal control of vascular tone through modulating the sensitivity of presynaptic β-adrenergic receptors and the balance between TXA_2 and PGI_2. The thesis also shows that in male rats, physiological concentrations of testosterone inhibit inward remodeling induced by chronic vasoconstriction. Mechanisms other that inactivation of TG2 seem to be involved in this inhibition.

RESUMEN

El objetivo de esta tesis fue analizar el papel de las hormonas sexuales masculinas en el control neuronal del tono vascular y en el remodelado vascular en la arteria mesentérica de rata, analizando por tanto los dos mecanismos que determinan en mayor medida las resistencias periféricas vasculares: el tono y la estructura vasculares.

Con respecto al tono vascular, trabajos previos han puesto de manifiesto que la ausencia de hormonas sexuales masculinas no modifica la contracción inducida por estimulación eléctrica (EE) en la arteria mesentérica de rata. Sin embargo, la contracción inducida por la administración de noradrenalina (NA) exógena es menor en arterias procedentes de animales castrados, sugiriendo un aumento de la liberación del neurotransmisor en ausencia de hormonas sexuales masculinas. Por otra parte, trabajos previos han mostrado que los estrógenos modulan la función de los receptores β-adrenérgicos, y éstos a su vez facilitan la liberación de NA y óxido nítrico (NO), por lo que se puede especular que los andrógenos también regulen la función de estos receptores.

Estudios previos han mostrado que la orquidectomía modula también la liberación y función de tromboxano A_2 (TXA_2), aunque el papel de este prostanoide en la respuesta contráctil inducida por EE no se conoce todavía. Por ello se analizó el efecto de las hormonas sexuales masculinas endógenas sobre la participación del TXA_2 en la respuesta vasoconstrictora inducida por EE y sobre la liberación de TXA_2 inducida por EE.

Las hormonas sexuales también modulan la estructura vascular. Sin embargo, el efecto de éstas sobre el remodelado hacia el interior (inward remodeling), no ha sido estudiado. Por tanto se analizó el posible efecto preventivo de la testosterona sobre el remodelado hacia el interior inducido por vasoconstricción sostenida en arterias mesentéricas de resistencia de ratas macho. Puesto que se ha demostrado que la enzima transglutaminasa 2 (TG2) está implicada en este tipo de remodelado, se analizó su participación en el efecto de la testosterona sobre el remodelado.

En los estudios sobre el tono vascular, se utilizaron segmentos desendotelizados de arteria mesentérica de ratas macho controles y orquidectomizadas de la cepa Sprague–Dawley. En ellos se analizó el efecto de la orquidectomía sobre la activación de los receptores β-adrenérgicos en la respuesta vasoconstrictora inducida por EE. Se midió también la liberación de NA y NO neuronal inducida por EE, analizando el papel de los receptores β-adrenérgicos y del TXA_2 en la liberación de dichos neurotransmisores. Asimismo, se analizó la liberación de TXA_2 inducida por EE, así como el efecto de este prostanoide sobre la liberación de prostaglandina I_2 (PGI_2) inducida por EE en ambos grupos de animales.

Los resultados obtenidos muestran que la orquidectomía no modifica la liberación de NA en respuesta a la EE, pero si incrementa la liberación de TXA_2, lo cual explica el mantenimiento de la respuesta vasoconstrictora inducida por EE a pesar de que la sensibilidad a la NA está disminuida en segmentos de animales orquidectomizados. La activación de los receptores β-adrenérgicos incrementa la liberación de NA y NO sólo en segmentos de ratas orquidectomizadas, confirmando que las hormonas sexuales masculinas modulan la función de los receptores β-adrenérgicos. La orquidectomía también modifica el papel del TXA_2 endógeno sobre la respuesta contráctil inducida por EE, afectando al menos a dos mecanismos, la liberación y respuesta al NO y la liberación de PGI_2. En animales controles, el TXA_2 incrementa la contracción inducida por EE, mediante la disminución de la liberación y la respuesta al NO. En animales orquidectomizados la respuesta contráctil inducida por EE permanece inalterada tras la inhibición de la formación del TXA_2, debido a un incremento en la liberación de PGI_2 que tiene un efecto vasoconstrictor.

En el estudio de remodelado vascular se utilizaron arterias mesentéricas de resistencia de ratas Wistar. La testosterona indujo una respuesta vasodilatadora dosis-dependiente que fue inhibida por la incubación previa con el inhibidor de la síntesis de NO, N_ω-Nitro-L-arginine methyl éster hydrochloride (L-NAME), confirmando que la testosterona induce la liberación de NO. El mantenimiento de la las arterias presurizadas, en medio de cultivo durante 3 días, e incubadas con endotelina (ET-1) indujo remodelado hacia el interior, que fue significativamente menor cuando las arterias se incubaron en presencia de testosterona. El remodelado vascular inducido también disminuyó significativamente por la presencia del inhibidor de la TG2, L682.777. La actividad TG2 no se modificó por la ET-1 y a su vez la testosterona no modificó este efecto.

Estos resultados muestran que la testosterona inhibe el remodelado hacia el interior inducido por vasoconstricción crónica a través de mecanismos distintos de la inactivación de TG2.

En definitiva, esta tesis muestra que la función vascular está influenciada por la función gonadal. La orquidectomía modifica el control neuronal del tono vascular mediante la modulación de la sensibilidad de los receptores β-adrenérgicos presinápticos y el balance entre el TXA_2 y la PGI_2. Esta tesis muestra también que concentraciones fisiológicas de testosterona inhiben el remodelado vascular inducido por la vasoconstricción crónica y que esta inhibición no parece ser debida a la inhibición de la actividad de la TG2.

1

GENERAL INTRODUCTION AIMS AND OUTLINE

CARDIOVASCULAR DISEASE

Cardiovascular diseases are the major cause of early mortality and morbidity in industrialized countries [1]. In the European Union, they account for 42 percent of all deaths in the total population [2]. Lifestyles are often affected by these diseases, resulting in disability and/or deterioration in quality of life. Because of the impact of cardiovascular disease, national and European health goals and targets have been developed and are currently being implemented to study the biological mechanisms underlying cardiovascular diseases.

Many risk factors associated with cardiovascular events have been established in population analyses. These include metabolic factors (total cholesterol, HDL cholesterol, fasting blood glucose), biological factors (blood pressure) and lifestyle factors (tobacco smoking, sedentarism, diet), all of which are modifiable, beyond the non-modifiable factors, such as age and male gender [3]. It is well known that diseases of the circulatory system are more common at advanced ages; in Europe, 81 percent of male deaths and 94 percent of female deaths due to this disease occur in subjects over 65 years [2]. Male gender is also considered a risk factor for cardiovascular disease, but the reason for this is not completely understood. Supported by the fact that clinical and epidemiological data show that the prevalence of cardiovascular diseases is higher in men compared to age-matched women [4] (Figure 1), the idea that male hormones were deleterious while female sex hormones were cardioprotective has been long time established in the scientific community [5-7]. However, the inverse correlation between cardiovascular disease and low serum levels of androgens in men has been addressed several times [8-10] and currently, it is widely accepted that both male and female sex hormones are vascular protective in their respective genders [11-18]. In women, the prevalence of diseases of the vascular system increases notably after menopause [17,19].

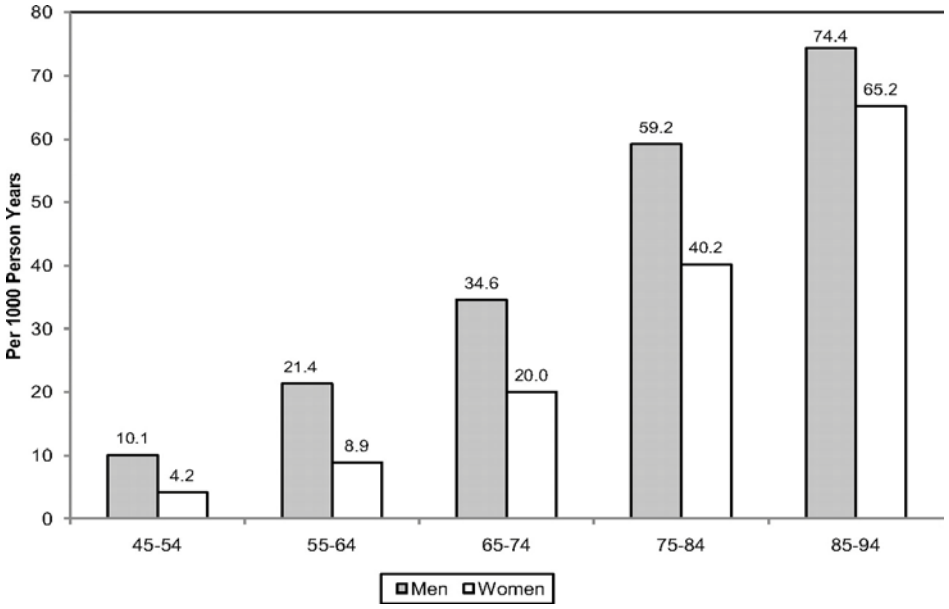

Figure 1. Incidence of cardiovascular disease* by age and sex (FHS, 1980–2003). *Coronary heart disease, heart failure, stroke, or intermittent claudication. With permission from Roger *et al*. [4].

Vascular diseases are also strongly associated with increased blood pressure [20, 21] because this situation lowers blood flow to certain tissues by increasing vascular resistance. Factors determining vascular resistances are described by the formula below, which is the result of combining Ohm´s law (pressure= flow x resistance) with the Poiseuille- Hagen formula,

$$R=\frac{8\eta L}{\pi r^4}$$

R = resistance
Ƞ = viscosity
r = radius of tube
L = length of tube

Considering that vascular length can be regarded as constant in most beds and blood viscosity is constant under most conditions and since flow varies directly and resistance inversely with the fourth power of the radius, blood flow and resistance in vivo are markedly affected by small changes in the calibre of the vessels [20, 22]. Thus, lumen diameter strongly determines the functional participation of arteries, as it determines their resistance to blood flow. Hence, the increase in vascular resistance is mainly due to a decrease in the vascular calibre, that is, a general narrowing of the vessel.

Modulation of vascular calibre is determined by both vascular tone and structure. Currently, it is becoming clear that vascular tone and vascular structure are highly

interrelated so that changes in tone affect the structure of the vessel and vice versa [23, 24]. The present thesis analyzes the effect of sex hormones on mechanisms regulating vascular tone and vascular structure, which are the two factors that determine vascular calibre, and thus vascular resistance and blood flow.

The splanchnic circulation, composed by the vessels supplying the abdominal viscera, plays a critical role in the control of the systemic blood pressure, as it collects the biggest volume of blood - 60% of the cardiac output - available in the circulatory system [25], so mesenteric arteries are important contributors to global vascular homeostasis.

GENERAL STRUCTURE OF THE VASCULAR WALL

Arteries are anatomically and functionally organized in three layers of tissue; intima, media and adventitia (Figure 2). Each one exerts its own influence on both the vasomotor control and the vascular structure, although the final effect is the result of the interrelated participation of the three layers. The anatomical organisation of the artery vessel wall was fully described by Rhodin [26].

The tunica intima delimits the vessel wall towards the lumen of the vessel and comprises the endothelium consisting of a simple and plain epithelium and its corresponding basal lamina of connective tissue. Above the connective tissue, it can be found the internal elastic lamina, which delimits the tunica intima from the tunica media. The endothelium plays a crucial role on the control of the vascular function, as it is not only a mechanical barrier, but also acts as a receptor and transmitter of signals between blood and other components of the vascular wall. Endothelial cells have exocrine, paracrine and autocrine effects, and they are involved in the regulation of vascular tone, vasculogenesis and angiogenesis, blood coagulation, fibrinolysis, and inflammation [27]. Endothelial cells are sensitive to hemodynamic changes such as pressure or shear stress forces, and to circulating chemical messengers. They respond to these signals by secreting different growth factors and vasoactive substances, among which it is worth noting vasodilator factors such as nitric oxide (NO) and prostacyclin (PGI_2) which also inhibit platelet aggregation [28], and endothelium derived hyperpolarizing factor (EDHF) [29]. Main vasoconstrictor factors released from the endothelium are endothelin-1 (ET-

1) and vasoconstrictor prostanoids such as thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2)[27, 30, 31].

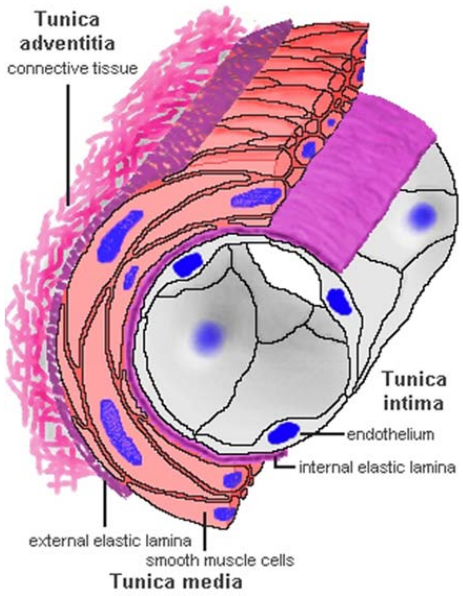

Figure 2. General structure of the artery vessel wall. From http://www.lab.anhb.uwa.edu.au/mb140/corepages/vascular/vascular.htm

The tunica media is formed by a layer of circumferential smooth muscle and variable amounts of connective tissue that forms the extracellular matrix. A second layer of elastic fibres, the external elastic lamina, is located over the smooth muscle. It delimits the tunica media from the tunica adventitia. Smooth muscle cells can also release different vasoactive substances [26, 32].

The tunica adventitia consists mainly of connective tissue fibres. The tunica adventitia blends with the connective tissue surrounding the vessel. This layer can be fenestrated to facilitate blood irrigation to the vessel wall through the *vasa vasorum*. Adventitia also receives and fixates the neuronal axons that innervate the muscular tissue, also called perivascular nerves [26, 33]. The adventitia also plays an active role in the regulation of vascular structure and tone through the release of factors such as fatty acids, different adipokines [34, 35] such as leptine [36], adipose derived relaxing factor (ADRF) [37], and reactive oxygen species (ROS) [38, 39].

MECHANISMS REGULATING VASCULAR TONE

Vascular tone is determined by an equilibrium in which endothelial factors, smooth muscle cells and innervation play important roles. In the mesenteric artery, which strongly modulates the control of global peripheral resistance, vascular tone is chiefly driven through a dual regulation mechanism involving both the endothelial and neuronal systems [40]. The endothelium may play a greater role in the response of blood vessels to local changes, while perivascular nerves may participate mainly in the integrated regulation of blood flow with the whole organism.

Nitric oxide

NO was first discovered in 1987 as a new second messenger [41] and then in 1990 it was also described as a new neurotransmitter [42]. It is one of the most widely studied factors in the regulation of vascular tone, and the mechanisms driving its synthesis, release, metabolism and mode of action are very well known [30, 43]. NO is a gaseous free radical that plays a vital role as a cell signalling molecule in the vascular, nervous and immune systems [44]. It is synthesized by enzymes called nitric oxide synthases (NOS), from L-arginine, which is oxidized to L-citruline using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor [45]. Three different isoforms of NOS have been described in mammals, coded from different genes, localized in different chromosomes, and functionally catalogued into constitutive or inducible expressed [46]. There are two constitutive isoforms the activity of which is dependent on intracellular Ca^{2+} levels. One of them, endothelial NOS (eNOS), is present in the endothelium, endocardium and myocardium, and the other, neuronal NOS (nNOS), is primarily present in the central and peripheral nervous system and also in cardiomyocytes [47]. The third isoform, inducible NOS (iNOS), is a high-output Ca^{2+}-independent NOS, the expression of which can be induced in a wide range of cells and tissues by cytokines or bacterial endotoxins [48].

Under physiological conditions, NO produced by constitutive NOS regulates vascular permeability and blood flow [49]. Thus, NO is not only released following stimulation, but also plays an important role in the maintenance of basal vascular tone, as demonstrated by the fact that infusion of an NOS inhibitor, L-NG-monomethyl arginine (L-NMMA), increases resting blood pressure [50]. NO is also an anti-proliferative, anti-inflammatory

and anti-thrombotic agent [51]. Under inductive conditions, high levels of NO synthesized by iNOS in activated immune cells can mediate anti-bacterial and anti-tumor functions, thus participating in the immune response [52].

Released NO diffuses to the smooth muscle cells where it stimulates soluble guanylate cyclase, resulting in an increased formation of cyclic guanosine monophosphate (cGMP) [53]. cGMP activates protein kinase G (PKG), which prevents the entry of Ca^{2+} into the cytoplasm by inhibiting the L type-Ca^{2+} channels in the cell membrane and activating the Na^{+}/Ca^{2+} pump. PKG also activates the Ca^{2+}-ATPase form in the cell membrane and the endoplasmic reticulum, to decrease intracellular Ca^{2+} concentration [54]. Besides, cGMP induces other actions that indirectly prevent increases in intracellular Ca^{2+} concentration, such as the activation of potassium (K^{+}) channels that hyperpolarize the cell membrane [55, 56] (Figure 3).

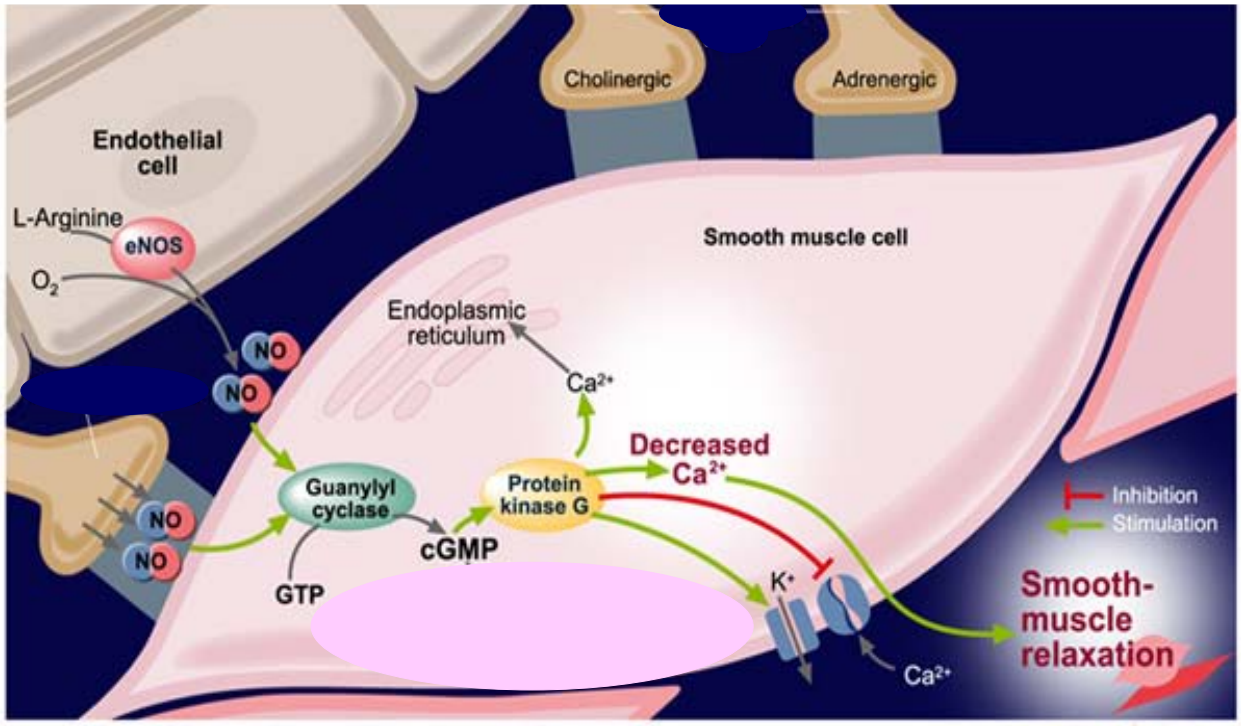

Figure 3. Schematic representation of the mode of action of nitric oxide in the vessel wall. NO can be produced under physiological conditions by the nitregic nerve endings through neuronal NOS (nNOS) or by endothelial cells through endothelial NOS (eNOS). Released NO diffuses to the smooth muscle cells where it stimulates soluble guanylate cyclase, resulting in an increased formation of cyclic GMP (cGMP). cGMP activates protein kinase G (PKG), which promotes relaxation of the smooth muscle cells through different mechanisms, including activation of Ca^{2+}-ATPase channels from plasmatic cell membrane and endoplasmic reticulum, promoting a decrease in the intracellular Ca^{2+} concentration; and activation of the membrane potassium (K^{+}) channels, which mediate membrane hyperpolarization. Adapted with permission from http://www.epgonline.org.

Innervation

Although research on the physiologic and pathologic mechanisms of vascular tone regulation, especially in the study of the role of sex hormones, have traditionally focused on the endothelial function, there is a great amount of evidence supporting the important role of innervation on the vascular homeostasis [32, 57-59]. Indeed, it has been hypothesized that under intensive vasoconstriction conditions, some neuronal dependent vasodilator mechanisms may be more important than endothelial-dependent ones [60]. This relevant participation of nerves gave rise to the concept of the dual control of vascular tone by both endothelium and neuronal mechanisms. As such, research on the role of sex hormones on vascular function should tackle this question.

The density of vascular innervation varies considerably between vascular beds in different tissues and between different physiological situations [30,61]. Usually, innervation density increases as the calibre decreases, so that small arteries possess the most intense innervation. The mesenteric artery, which strongly modulates global peripheral resistance, is densely innervated. Nerves surround blood vessels forming a complex grid-like network termed the perivascular nerve plexus (Figure 4). Perivascular nerves do not penetrate smooth muscle cells, but they stay within the media and the adventitia [62]. Innervation of arteries is composed of nerves from the autonomic neuronal system, consisting mainly in sympathetic, parasympathetic and sensory-motor nerves [30]. In the mesenteric artery, sympathetic innervation is composed of adrenergic nerve terminals releasing mainly noradrenaline and producing vasoconstriction [63]. Sensory-motor nerves are primarily afferent sensory nerves that also release neuropeptides, mainly calcitonin gene-related peptide (CGRP), resulting in vasodilation [64, 65]. Parasympathetic innervation has a vasodilatory effect and is composed of both the cholinergic and nitrergic nerves releasing mainly acetylcholine (Ach) and NO respectively [53]. The net nervous effect on the vascular tone is the result of the integration of vasoconstrictor and vasodilator influences. Thus, electrical stimulation of nerve endings in the mesenteric artery produces a vasoconstrictor response that is the integrated result of the release and response to different neurotransmitters, mainly NA, NO and CGRP [53, 63, 64].

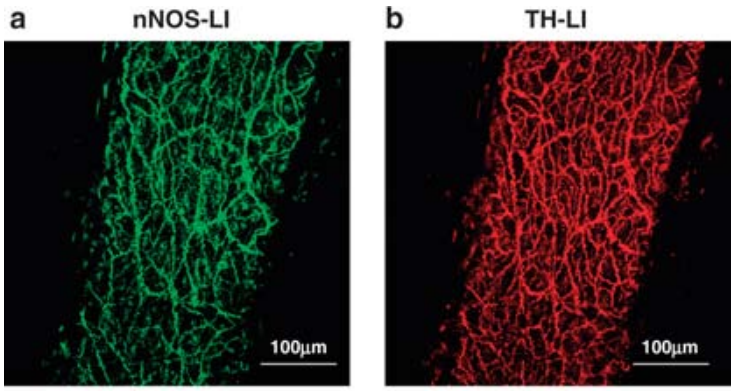

Figure 4. Confocal laser photomicrographic images showing perivascular nerves in the rat mesenteric artery. Images show; (a) the neuronal nitric oxide synthase (nNOS)-like immunoreactivity (LI) in green and (b) tyrosine hydroxides (TH)-LI-containing fibers (adrenergic fibers) in red. The horizontal bars indicate 100 μm. Modified with permission from Koyama *et al*. [84].

Autonomic neuro-effector junctions in blood vessels are atypical, as they contain pre-junctional membrane thickenings with synaptic vesicles, but without specialized post-junctional structures. This is probably related to the fact that the periarterial nervous plexus is not a fixed network, as it can undergo dynamic changes in response to physiologic or may be pathologic changes [66]. Besides, nerves usually contain neurotransmitters and cotransmitters that interact and modulate each other, often involving synergistic cotranssmision or pre- and post-junctional neuromodulation of neurotransmitter release [33, 66].

Adrenergic innervation

The sympathetic control of the vascular resistances has a paramount role on the control of the peripheral vascular resistance [67]. Stimulation of sympathetic nerves in the splanchnic circulation increases peripheral resistance and mobilizes up to two thirds of the reserve blood volume in the veins [68]. Besides, one of the major pathophysiological mechanisms of hypertension includes activation of the sympathetic nervous system [69]. The activity of this innervation, primarily vasoconstrictor, over the arterial wall is exerted by the release of NA. NA release from the adrenergic nerve terminals seems to be the major contributor to the contraction induced by electrical field stimulation, as preincubation with the α-adrenoceptor antagonist phentolamine strongly inhibits this response [70].

Adenosine triphosphate (ATP) and neuropeptide Y (NPY) are cotransmitters released with NA in these nerves, and they also mediate vasoconstriction [33, 71]. However, its

role in the adrenergic-induced contraction does not seem to be very relevant in the rat mesenteric bed, as preincubation with the blocker of nerve impulse propagation tetrodotoxin, inhibits the contraction induced by electrical field stimulation to a similar extent as phentolamine does [70].

Upon release, NA binds post-synaptic α_1 and α_2-adrenoceptors on arterial smooth muscle cells, leading to contraction [33]. Stimulation of post-junctional α_1-adrenoceptors activates the phospholipase C (PLC) through a G protein. PLC catalyzes the transformation of phosphatidyl-inositol bisphosphate (PIP_2) in diacylglycerol (DAG) and inositol triphosphate (IP_3), resulting in the release of the stored Ca^{2+} and the activation of the protein kinase C (PKC) respectively, thus producing contraction of the smooth muscle cells [72, 73] (Figure 5). Stimulation of post-junctional α_2-adrenoceptors inhibits the enzyme adenylate cyclase, thus decreasing the cyclic adenosine monophosphate (cAMP) levels, which also increases the cytoplasmatic Ca^{2+} concentration. Simultaneously, NA can activate pre-junctional α_2-adrenoceptors, which mediate inhibition of NA release in a negative feedback mechanism [74, 75] (Figure 6).

NA can also bind to pre- or post-synaptic β-adrenoceptors. Activation of the former facilitates NA release, thus consisting of a positive feedback mechanism [76, 77]. Activation of post-synaptic β-adrenoceptors induces relaxation of the smooth muscle cells (Figure 6). These receptors are coupled to G proteins that activate the enzyme adenylate cyclase thus inducing cAMP formation, which leads to the relaxation of the smooth muscle cell [78]. Different subtypes of β-adrenoceptors have been identified. Among them, β_1-adrenoceptors have high affinity for noradrenaline and are predominantly found in heart, brain and adipose tissue, while β_2-adrenoceptors have low affinity for noradrenaline and are present in the vascular smooth muscle cells [73]. More recently, the existence of functional vasodilatory β_1-adrenoceptors in the vascular wall has also been addressed [79]. β_3-subtype adrenoceptors have also been described although it has been reported that they are not present in the rat mesenteric artery [79]. However, the net effect of adrenergic receptor activation in arteries is vasoconstriction, which has been explained by the fact that NA has more affinity to α- than to β-adrenoceptors, and also because of the increased presence of α- compared to β-adrenoceptors in the vascular smooth muscle cells [63].

There is great heterogeneity among the subtypes of adrenoceptors present in different vascular beds, differing even between strains. But there are also many differences in the functional role of adrenoceptors between distinct physiological situations [59, 80, 81] supporting the idea of the mobile and adaptative nature of the periarterial nervous plexus commented above [66].

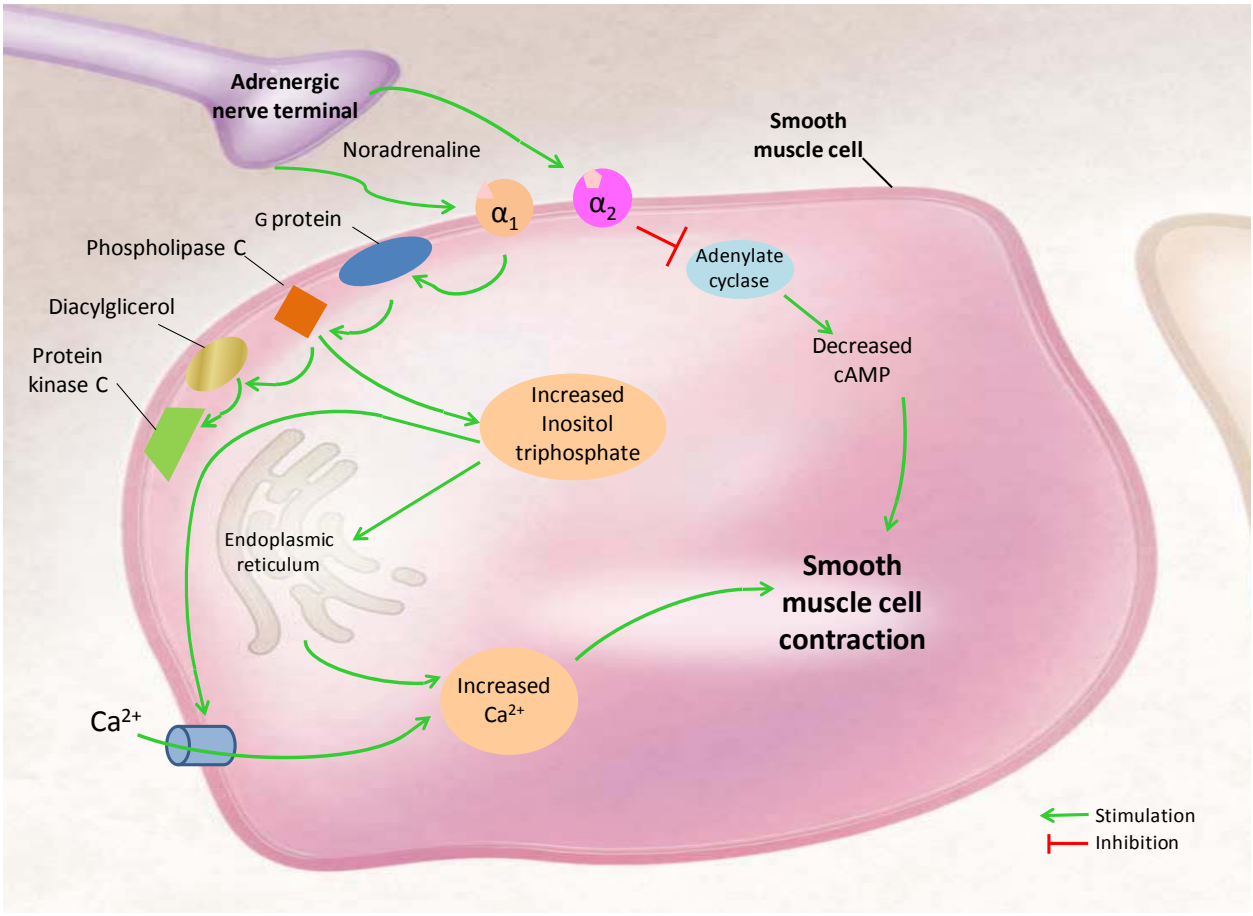

Figure 5. Molecular mechanism of adrenergic-mediated smooth muscle contraction. Norepinephrine from sympathetic nerve endings activates receptors on smooth-muscle cells to initiate the cascade of reactions that results in elevation of intracellular calcium concentrations and smooth muscle cell contraction. Protein kinase C is a regulatory component of the calcium-independent, sustained phase of agonist-induced contractile responses. Adapted with permission from Lue [82].

Nitrergic innervation

The mesenteric artery possesses perivascular nitrergic innervation that releases NO in response to electrical stimulation [83], thus participating in the regulation of vascular tone. Because of its chemical characteristics, NO does not have a receptor, as it has a half life of 5 seconds and it is a gaseous, small, polar, uncharged molecule. These characteristics allow it to diffuse inside the adjacent cells, where it exerts its function through stimulating soluble guanylate cyclase, as explained previously (Figure 3).

Some authors have suggested that neurogenic NO seems not to have a very significant role in neurogenic vasodilatation in the rat mesentery [33, 53], although nitrergic nerves have been inmunohistochemically detected [84](Figure 4) and inhibition of neuronal NO release by L-NAME in endothelium denuded mesenteric arteries has also been shown to increase the contractile response induced by electrical field stimulation [70, 83]. Besides, the release of neuronal NO induced by EFS has been demonstrated, since tetrodotoxin, which blocks the nerve impulse propagation, abolishes this release [85]. It has been suggested that NO release could be up-regulated by pre-junctional activation of β-adrenoceptors by NA in the nitrergic nerve endings [83] (Figure 6).

Sensory innervation

Previous reports provided evidence that periarterial nerve stimulation of arteries produces non-adrenergic non-cholinergic neurogenic vasodilation, which is mediated by CGRP [86, 87] (Figure 6). Upon release, CGRP binds to its functional receptor consisting of receptor-affinity-modifying protein 1 (RAMP1) associated with calcitonin receptor-like receptor (CRLR) [88]. CGRP receptors are present in endothelial cells, where they potentiate NO and PGI_2 formation and; also in smooth muscle cells, where they activate adenylate cyclase increasing cAMP levels and consequently induce relaxation [89, 90].

Sensory nerves can suppress sympathetic nerve-mediated vasoconstriction by releasing CGRP and conversely, sympathetic nerves presynaptically inhibit the release of CGRP [64, 91] (Figure 6). Thus, it has been proposed that vasodilator sensory nerves, along with vasoconstrictor sympathetic nerves, regulate the tone of the mesenteric resistance arteries. Nevertheless, previous studies carried out by my research group showed that sensory innervation does not have a role in the

vasoconstrictor response to electrical field stimulation in the rat mesenteric artery [70, 83]. In contrast, a functional role for sensitive innervation has been demonstrated using the same methodology in diabetic [58], and in hypertensive male rats[92], but not in hypertensive female rats [57]. All these data support the idea that the participation, and thus the physiological relevance of sensory-motor innervation varies depending on the strain, the physiological situation, and even on gender.

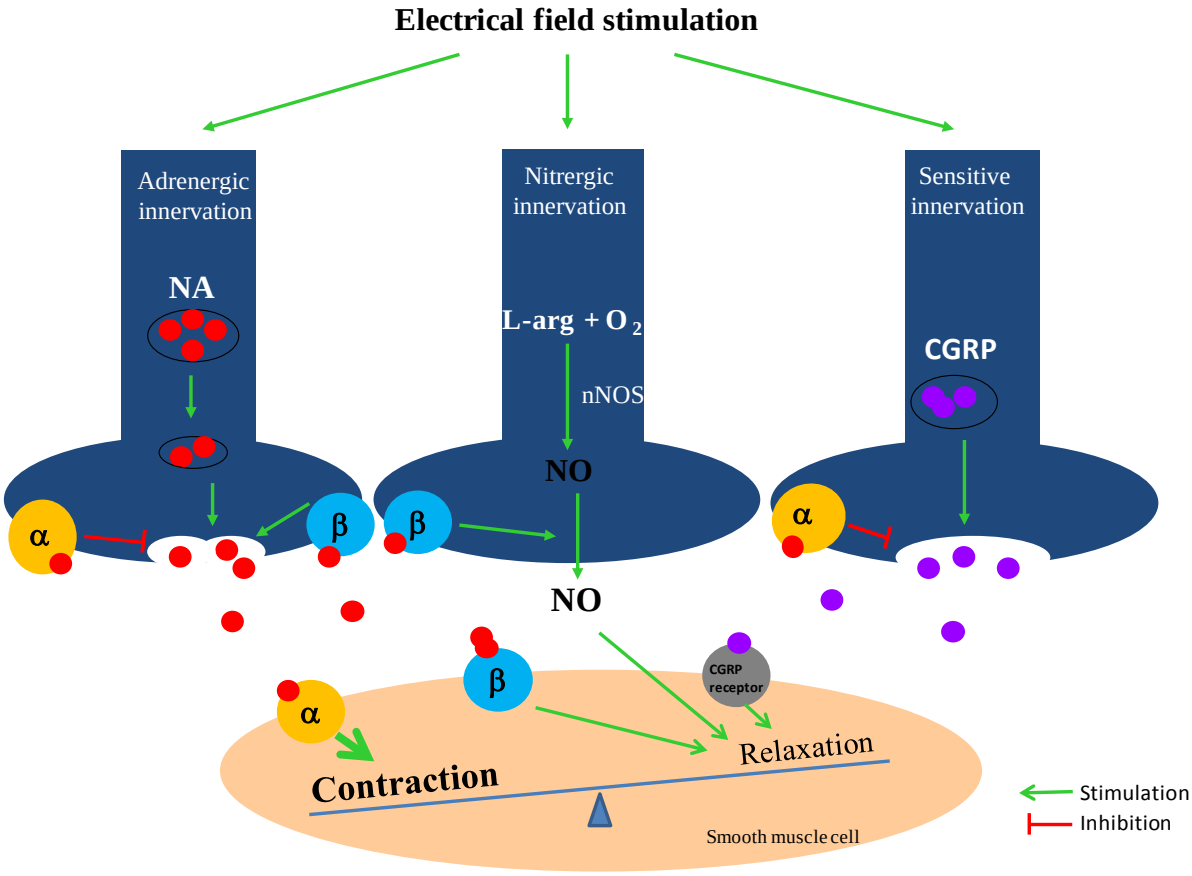

Figure 6. Factors influencing the contractile response induced by electrical field stimulation. EFS applied to the mesenteric artery induces a contractile response that is the integrated result of the release of noradrenaline (NA), nitric oxide (NO) and calcitonin gene-related peptide (CGRP) from adrenergic, nitrergic and sensory innervations respectively.

Cholinergic innervation

Ach release from parasympathetic nerves results in activation of muscarinic receptors on vascular smooth muscle cells, inducing contraction of these cells and vasoconstriction, but Ach release also activates muscarinic receptors on endothelial cells producing NO release that leads to vasodilation [93]. ATP and vasoactive intestinal

polypeptide (VIP) have been shown to be co-transmitters with Ach in parasympathetic nerves [71]. However Ach containing nerves are not ubiquitously present and it is thought that this innervation has very little effect or is even absent in most peripheral resistance arteries [33, 53, 94]. In fact, stimulation of cholinergic nerves in the mesenteric bed does only produce a small vasodilatation [60]. Therefore it does not seem to have relevance in the control of peripheral resistance. Nevertheless, cholinergic receptors are ubiquitously expressed in the arterial wall, and endothelial cells express the enzymatic machinery for the synthesis and transport of Ach, as well as acetylcholinesterase, thus pointing to the existence of an additional intrinsic, non-neuronal cholinergic system residing in the vascular wall [95]. However, the functional relevance of this system remains to be defined.

Prostanoids

Prostanoids are metabolites from arachidonic acid very well known for their pivotal functions in cellular processes such as inflammation, platelet aggregation and vascular tone regulation [96]. They are present in almost all cell types and are involved in many vascular pathologies. NO and prostanoid systems are known to modulate each other, although modulation of the NO systems by prostanoids has been less studied [97-101].
Prostanoids are synthesized from arachidonic acid (AA) which is in turn formed from membrane phospholipids through the enzymes phospholipase A_2 (PLA_2) and diacylglycerol lipase. AA is converted to the prostanoid precursor prostaglandin G_2 (PGG_2), which is subsequently peroxidized to prostaglandin H_2 (PGH_2). Both enzymatic reactions are catalyzed by the protein cyclooxygenase (COX); its cyclooxigenase activity adds two O_2 molecules to the AA generating PGG_2, whereas the peroxidise activity catalyzes the reduction of PGG_2 to PGH_2 [102] (Figure 7). There are two physiologically relevant isoforms of COX: COX-1 is constitutively expressed and localized in the endoplasmic reticulum, and COX-2 is inducible and primarily localized in the nuclear membrane [103]. Induction of COX-2 occurs in certain cell types in response to proinflammatory stimuli, and hence most of the inflammatory functions of prostanoids synthesis are attributed to this isoform of COX [96, 104]. A third isoform of COX, called COX-3, has been discovered in dogs [105], but it is currently not thought to have a physiologically relevant role on prostaglandin synthesis in rodents and humans[106].

Bioactive prostanoids are prostaglandin E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$), D_2 (PGD_2), I_2 (PGI_2), and thromboxane A_2 (TXA_2). They are formed from PGH_2 through their respective synthases (Figure 7) [107]. Each prostanoid exerts its action by binding to its specific receptor (Figure 7). However, depending on the cell type, the physiologic situation or the concentration, ligand-binding can be observed between a given prostanoid and other receptors within the family [96]. In this situation, a prostanoid can produce an effect opposite to that usually described.

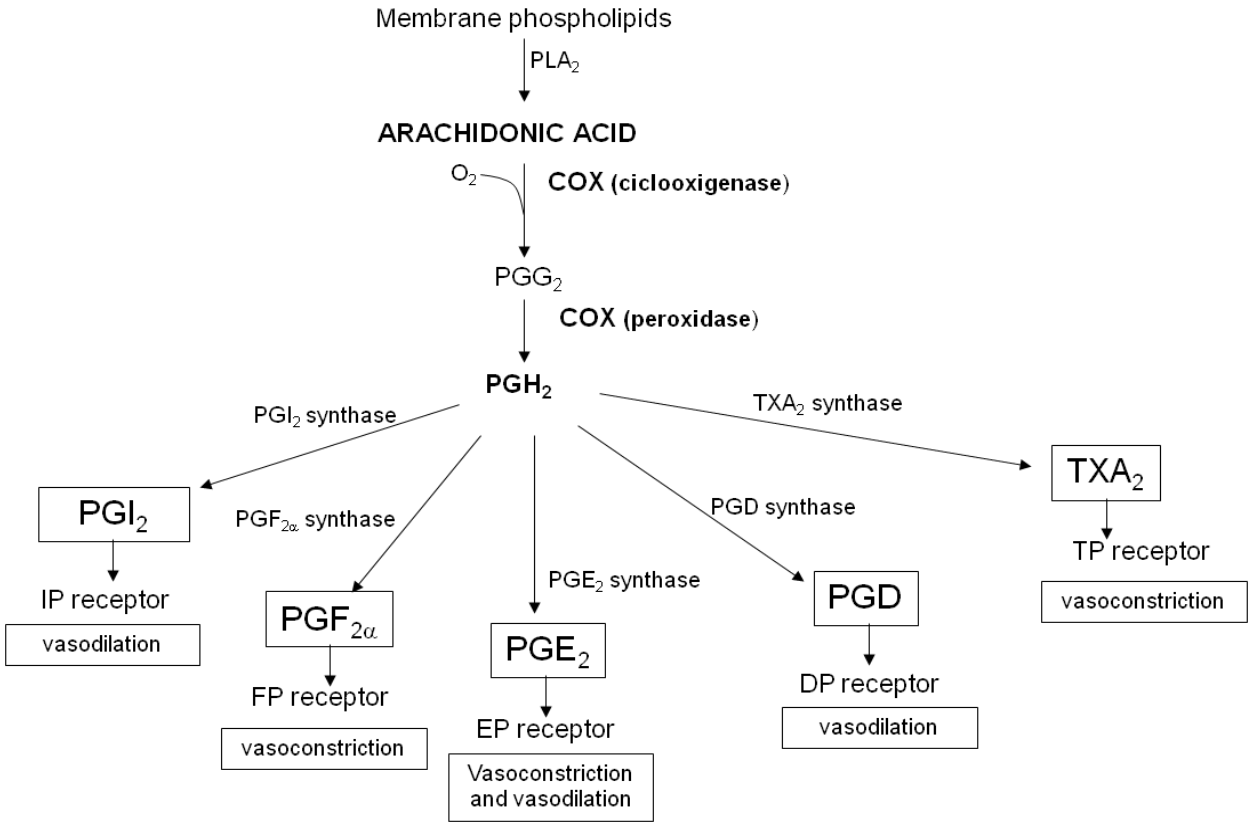

Figure 7. Synthesis pathway of the different bioactive prostanoids from arachidonic acid through the enzymatic activity of COX. For each prostanoid, specific receptors and their vasomotor effect upon activation are indicated.

Thromboxane A_2

TXA_2 is one of the prostanoids produced in the vascular wall that has been most studied, due to the importance of its important role in vascular homeostasis. Both endothelial [108] and smooth muscle cells [109] synthesize it. TXA_2 is a potent vasoconstrictor and inductor of platelet aggregation and it is involved in several pathologies such as hypertension, atherosclerosis, myocardial infarction and stroke

[110, 111]. Thus, TXA_2 can participate in the endothelial dysfunction associated with cardiovascular disease [112]. TXA_2 activates its specific receptor, the TP receptor, which is functionally coupled to a G protein. The activation of these components inhibits adenylate cyclase, leading to a decrease in cAMP levels [111]. Activation of TP receptors also induces the activation of PLC. As commented above, PLC catalyzes the transformation of PIP_2 in DAG and IP_3, resulting in the release of stored Ca^{2+} and the activation of PKC respectively, and this leads to the contraction of the smooth muscle cells [72]. The activation of these signalling pathways induces not only platelet aggregation and vascular smooth muscle cells contraction but also hypertrophy and cell proliferation [96].

Prostacyclin.

Prostacyclin, or PGI_2, which is synthesized by the PGI_2 synthase [113], induces vasodilation and inhibits platelet aggregation on vessels [96]. PGI_2 can induce different or even opposite effects depending on its concentration and on the G protein to which the IP receptor is coupled. Thus, if the IP receptor is coupled to a G_s protein, it stimulates when activated, the production of cAMP by activating adenylate cyclase [114]. cAMP can act both directly and indirectly on ionic channels, finally producing a decrease in cytosolic Ca^{2+}, that leads to smooth muscle cell relaxation. PGI_2 can also induce vasodilation upon activation of the endothelial IP receptors, which induce NO release [115]. On the other hand, IP receptors can couple to G_q and G_i proteins [116], which would induce vasoconstriction. Besides, high concentrations of PGI_2 have been reported to induce vasoconstriction as a result of the activation of TP receptors [117, 118]. However, the general vasomotor mechanism of PGI_2 seems to be the counteraction of the effect of TXA_2. In fact, it has been described that PGI_2 can antagonize the effects of TXA_2, and it can even reverse hypertrophy induced by the TXA_2 analogue U-46619 [119].

Reactive oxygen species

The formation of ROS is a common feature of normal cell metabolism. ROS generally increase vascular tone through their influence on the endothelium, as well as by either direct effect on vascular smooth muscle contractility. One of the most important ROS in vascular function is superoxide anion (O_2^-) [120]. Superoxide anion is a negatively-

charged free radical that undergoes rather selective chemical reactions with components of biological systems. Superoxide anion is produced by the reduction of O_2 and is toxic for the cell when is present in high amounts. Because NO has an unpaired electron, it can react with O_2^- producing peroxynitrite ($ONOO^-$) as the result of a spontaneous and irreversible chemical reaction [120]. The consequence of this reaction is a decrease in NO bioavailability [121] thus attenuating its vasodilator effect, and the generation of peroxynitrite, that can potentially interact with regulatory systems of biological significance.

ROS can also contribute to vascular remodeling as they can modify the smooth muscle cell phenotype, and alter processes such as growth, migration, cell death, or extracellular matrix reorganisation [122]. Excessive ROS production or an insufficient antioxidant activity can induce alterations in vascular function.

VASCULAR REMODELING

Since 1987, when Glagov reported the surprising finding that atherosclerotic arterial lumen narrowing is not simply the result of enlargement of atherosclerotic lesions [123], the concept emerged that wall structure can change to maintain the appropriate lumen size to permit normal blood flow, and this was termed vascular remodeling [124].This ability of arteries to adapt is central to most arterial diseases. Nevertheless, the inability of vessels to remodel appropriately is considered a form of 'vascular failure' that can lead to pathologic states such as hypertension or atherosclerosis [125, 126].

Mulvany proposed a terminology to classify structural changes in hypertensive vessels based on changes in lumen diameter (inward or outward) and wall area (increased=hypertrophic, decreased=hypotrophic, no change=eutrophic) [127] (Figure 8). Irrespective of the mechanism responsible, the main feature of hypertensive vascular remodeling is the increased media/lumen ratio, which can occur with or without growth (i.e. hypertrophic or eutrophic). Patients and animal models with essential hypertension exhibit predominantly eutrophic inward remodeling, which allows the vessels to maintain an increased resistance without increasing vascular tone [128].

Global peripheral resistance is mainly determined by the resistance vessels, consisting of the small arteries (arteries with diameter < 300 μM) and the arterioles (the arteries just before the capillaries) [129]. Increased peripheral resistance is a common parameter in essential hypertension, and current evidence indicates that it is due in part to a general narrowing of the resistance vessels [130].

Inward remodeling of small vessels is characterized by a decrease in the outer and lumen diameter, and an increase in the media/lumen ratio [131], with no change in the wall cross sectional area. This vessels are stiffened, which means that compared to normal vessels, they are not able to increase its diameter in response to increases of pressure [132].

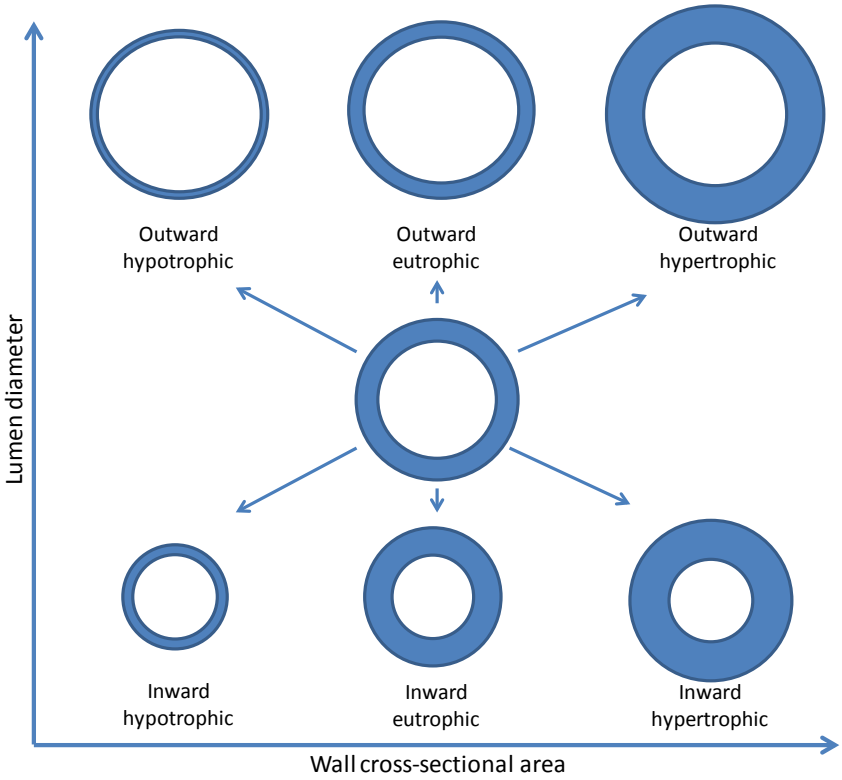

Figure 8. Patterns of vascular remodeling. Classification refers to changes on the lumen diameter (inward or outward) and vessel cross-sectional area (hypertrophic, hypotrophic or eutrophic). Adapted from Mulvany [127].

Vascular structure is studied with the smooth muscle cells relaxed, and usually with exposure of the vessels to a given intravascular pressure [24, 128]. Mechanisms involved in vascular remodeling have been investigated in organ culture, where vessels are cannulated and maintained under pressurized conditions during three days, retaining their viability [132]. In this series of studies, Bakker et al. have reported that maintained constriction is necessary for inducing remodeling [133, 134]. This remodeling is eutrophic, as it is not the result of any change in the cross-sectional wall area. Indeed, this remodeling could also be induced in the presence of growth inhibitors [134]. These experiments support the idea that eutrophic inward remodeling, could be the result of sustained vasoconstriction [24, 135]. In fact, the early stages of hypertension are thought to be associated with increased sympathetic activity (which mediates vasoconstriction, as explained below) and thus presumably increased vascular tone [136]. These authors suggested that eutrophic remodeling, which is seen in essential hypertension, is the result of the reorganization of the wall material during the constriction period, in such a way that the initial active narrowing of the lumen would become permanent and passive [133] (Figure 9). This hypothesis supports the idea of

the interrelation between vascular tone and structure [24]. Interestingly, active constriction of the lumen diameter is mandatory for inducing remodeling, since passive narrowing of lumen diameter (i.e. decreasing intravascular pressure), does not induce any remodeling and besides, vasodilatation prevents inward remodeling [133]. This idea correlates well with data showing a decrease in the remodeling process of hypertensive subjects after treatment with vasodilators, but not after reducing blood pressure [137].

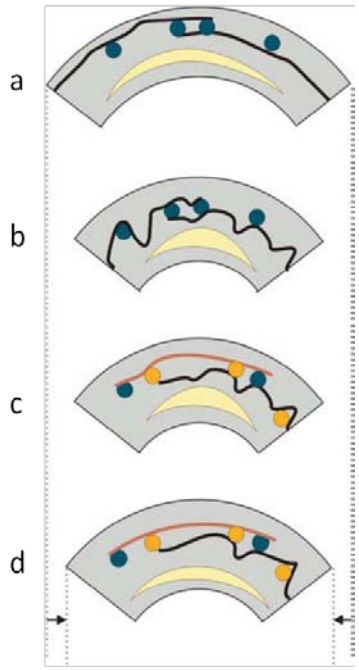

Figure 9. Schematic representation of the hypothesis that links vasoconstriction to inward remodeling. **a** Segment of the vessel wall in a relaxed state, showing smooth muscle cells in yellow, structural elements form the extracellular cell matrix in black, and cross-links in blue. **b** contracted vessels where the shortening of the SMC narrows the lumen. **c** during chronic vasoconstriction extracellular matrix reorganizes and forms new cross-links. **d** Upon vascular relaxation, passive forces from the newly organized extracellular matrix narrow the lumen, preventing dilation to the original diameter. With permission from van den Akker *et al*. [24].

Structures involved in the process described above are thought to be the components of the cytoskeleton along with membrane integrins of smooth muscle cells and elements of the extracellular cell matrix (ECM), particularly collagen and elastin. Integrins are proteins that connect the cytoskeleton with the ECM and also act as receptors. Collagen is mainly responsible for providing strength to the vessel wall, while elastin, is the major component of the elastic fibers. The quantity and quality of each one of these components determines in part the mechanical properties of vascular structure. Fibronectins are attached to integrins and can also modulate characteristics of the ECM [24, 135, 138, 139]. Regarding the hypothesis of the vascular tone driving inward

eutrophic remodeling, Martinez-Lemus et al. support that during sustained vasoconstriction, there may be a re-positioning of vascular smooth muscle cells over the reduced lumen diameter, and they confer a role to the cytoskeleton and integrins in the remodeling process [135].

It should be noted that although there is no change in the vessel wall mass in the eutrophic remodeling described, smooth muscle cell proliferation and apoptosis should not be discarded as part of the remodeling process [140].

Transglutaminase 2 in vascular remodeling

It has been recently reported that transglutaminase 2 (TG2) is involved in the inward remodeling of small vessels [141]. Thus, inward remodeling induced by low blood flow *in vivo*, or by chronic vasoconstriction *in vitro* can be inhibited by TG2 inhibitors [142]. Transglutaminases are a group of enzymes known for their ability to provide mechanical strength to the tissues by catalyzing protein cross-links [143]. The most important cross-link catalyzed by transglutaminases in vascular remodeling is the N^{ε}(γ-glutamyl)lysine isopeptide bond, which stabilizes the ECM. TG2 is involved in wound healing, cell adhesion, apoptosis and matrix reorganisation, and recent reports have suggested that it could also play a key role in vascular remodeling [141]. These authors proposed that TG2 could fixate the extracellular matrix into a more narrowed state during chronic vasoconstriction, through cross-linking its proteins. They found several extracellular substrates for TG2, including fibronectin, collagen and nidogen [144]. TG2 is ubiquitously expressed and as such, is present in smooth muscle cells, endothelial cells and vessel wall fibroblasts [145]. Its cross-linking activity can be activated by elevated intracellular Ca^{2+}, and inhibited by NO and guanosine triphosphate (GTP) [146, 147]. Therefore most of TG2 activity may be latent under physiological conditions. Overexpression of TG2 alone does not result in intracellular cross-linking [148], indicating that its effect depends on its activation. However, activation and translocation of the enzyme to the outer face of the vessel wall is necessary to induce remodeling [144], supporting the idea that TG2 would cross-link proteins from the extracellular matrix. However, the link between sustained constriction and TG2 translocation and activation remains unknown, although van den Akker et al. proposed that a reducing environment is necessary to induce membrane-bound activity of TG2 [144].

Of particular interest for the study on the effect of sex hormones on vascular remodeling included in this thesis, is the fact that TG2 can be inhibited by NO through S-nitrosylation of cysteine residues [147]. In fact, NO donors inhibit vascular remodeling induced by exogenous transglutaminase or by an intracellular reducing agent [142, 144]. Besides, animal models of hypertension induced by L-NAME intake, have inwardly remodeled small mesenteric vessels, an effect that was not induced in a transglutaminase knockout mice which received the same treatment with L-NAME [149]. Therefore, inward remodeling could be present in any pathology associated with decreased NO production or bioavailability due to the promotion of TG2 activation.

SEX HORMONES

Sex hormones produce a great variety of biological effects. They induce differentiation and cell growth of reproductive tissues, and development and maintenance of secondary sexual characters after the beginning of puberty [150].

Estrogens (estradiol, estrone and estriol) and progestagens (progesterone) are the main female sex hormones, while testosterone is the main male sex hormone, although some of its precursors and metabolites can also be of biological relevance [12, 151]. Sex steroids are mainly synthesized in gonads and secondarily in the suprarenal cortex from cholesterol (Figure 10) [152]. Nevertheless, production of endogenous estrogens and androgens from their precursor dehydroepiandrosterone (DHEA) has also been described in a number of extragonadal sites such as adipose, epithelial, osseous, neuronal and vascular tissues [153, 154]. Besides, testosterone can be converted to estradiol by aromatase in the extragonadal sites described above [154].

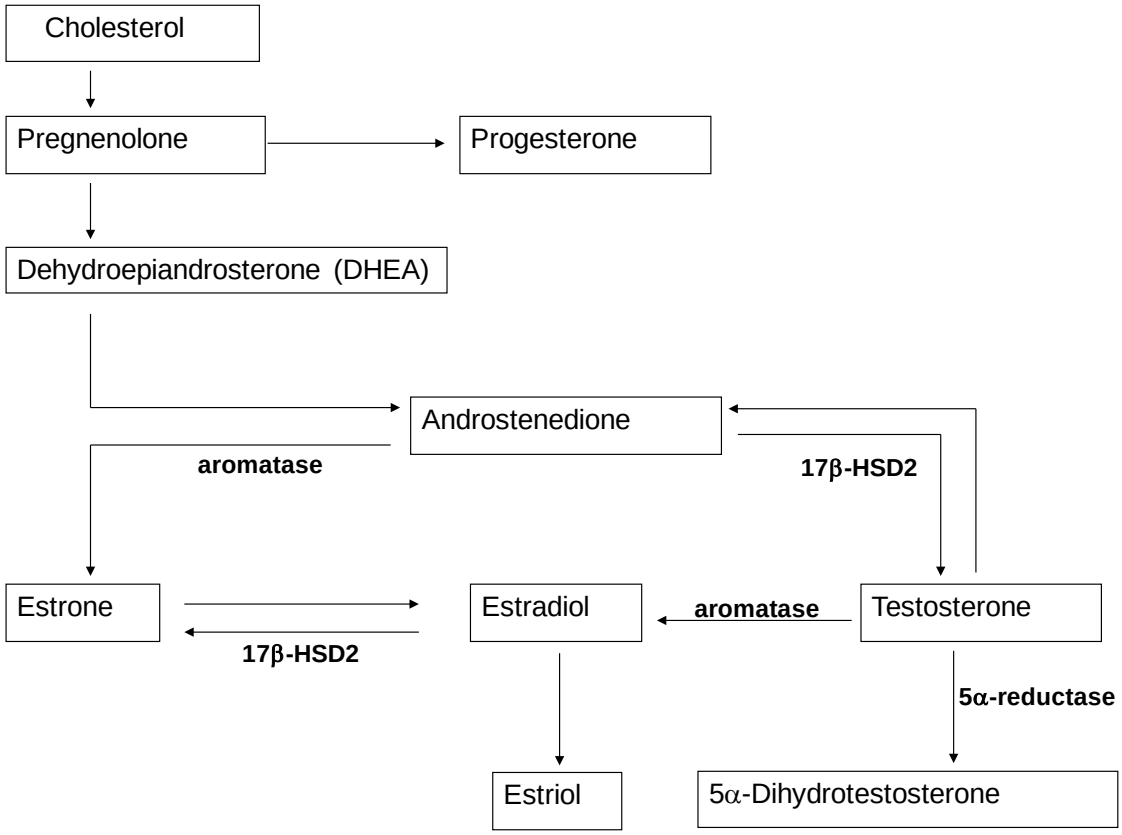

Figure 10. Conventional synthetic pathway of sex hormones. 17beta-hydroxysteroid dehydrogenase type 2 (17β-HSD2). Adapted from Ghayee and Auchus [152].

Findings in the last decades have shown that sex hormones play important roles on the vascular system [12-15], and hence low serum levels are independently related to cardiovascular disease in both genders [11, 16-18]. This protective effect of sex hormones can be attributed to their multiple modes of action, such as modulation of the lipid profile, vascular tone and vascular structure [13, 15].

Biological effects of sex hormones can be induced via genomic mechanisms, which involve steroid binding to its nuclear or cytosolic receptor, a ligand-activated transcription factor, thus modulating gene expression. The genomic effects of estradiol in vascular function include promoting endothelial cell growth and inhibiting growth of vascular smooth muscle cells [155]. Regarding androgens, some studies have suggested that they potentiate vascular smooth muscle cell growth while others suggest that they inhibit it [156, 157].

Sex hormones can also act without involving transcription, through non-genomic effects [158]. These effects can be triggered by receptor-dependent as well as receptor-independent mechanisms. For example, both estradiol and testosterone may induce vasodilation in a non-genomic manner by different pathways involving receptor dependent and independent mechanisms [12, 155]. However, sex hormones can activate different signalling pathways through non-genomic mechanisms that can indirectly lead to modifications on the gene expression.

Sex hormones and vascular tone

Both male and female sex hormones can induce rapid vasodilation in an endothelium dependent manner. Acute infusion of estrogen into arteries causes dilatation owing to both an increased release of nitric oxide from the endothelium and the inhibition of ion channels in the smooth muscle. Increased release of endothelial NO mediated by female sex hormones can be mediated by both genomic and non-genomic pathways: by rapid signalling with membrane estrogen receptors through the PI-3Kinase/Akt pathway resulting in eNOS phosphorylation, and also increasing eNOS expression in a long term genomic manner [159].

Androgens can also induce endothelial NO-mediated vasorelaxation [12] in a rapid non-genomic manner, although the mechanism underlying this effect has not yet been

established. Whether it activates the release of nitric oxide directly or indirectly through aromatization to estrogen remains controversial [15, 159], although some studies have specifically evidenced an androgen mediated vasodilation independent from the conversion to estrogens [160, 161]. Androgens can also induce rapid non-genomic vasodilation by modulation of the vascular smooth muscle cells ion channel function, particularly by inhibiting Ca^{2+} channels and activating K^{+} channels in the cell membrane [160, 162].

Neuronal modulation of vascular tone strongly determines peripheral vascular resistances. Despite this, the effect of sex hormones on these mechanisms has been less studied. In fact, many reviews on the protective effect of sex hormones on vascular function do not mention it [11-15]. There is, though, a recent review that specifically focuses on the effect of sex hormones on neuroeffector mechanisms, including vascular function [67].

Sex hormones influence the vascular adrenergic function [67, 163], although the mechanism by which this modulation occurs is still not fully understood. Positive correlations between androgens and parasympathetic activity have been described in men [164], and greater vascular resistance has been recorded in men compared to women, the difference being attributed to alpha-adrenergic activity [165]. In rat mesenteric arteries, orchidectomy decreases the contraction induced by exogenous NA, though the vasoconstrictor response to EFS is not modified [70]. Together all these data suggest that modulation of the adrenergic activity is one of the mechanisms by which androgens can alter peripheral vascular resistances. Female sex hormones also modulate vascular adrenergic activity, as it has been suggested that estrogens could limit the production of noradrenaline, attenuate the vasoconstrictor response to noradrenaline and increase vasodilator β-adrenoceptor sensitivity [67, 80].

Regarding nitrergic innervation, studies from my research group have shown that mesenteric arteries from orchidectomized rats have decreased nNOS expression, although the release of neuronal NO is not changed [70] because nNOS activity is increased through PKC activation [85]. However, NO metabolism is higher in segments from orchidectomized male rats due to the increased generation of superoxide anion (O_2^-) and peroxynitrite formation [70].

In females, the effect of endogenous sex hormones on the nitrergic innervation has also been studied in my laboratory. Results showed that NO release is increased in mesenteric segments from ovariectomized rats, and although NO metabolism is higher in this group, the net contribution of neuronal NO to the vasoconstrictor response induced by electrical stimulation is greater in ovariectomized animals [166], which was suggested to be a compensatory mechanism to counteract the loss of endothelial NO reported in the same artery [81].

Sex hormones also influence TXA_2 release and function. Hence, endothelium-mediated TXA_2 release is increased in arteries form orchidectomized and ovariectomized rats by increasing COX-2 expression [101, 167, 168]. Testosterone was also shown to increase TXA_2 vasoconstrictor response [169] and to increase the density of TP receptors in vascular smooth muscle cells [170, 171]. However, the effect of orchidectomy on TXA_2-induced contractile response depends on the vascular bed, since in the mesenteric artery castration did not modify the response to the TXA_2 mimetic U-46619, while it increased this response in aorta [167, 168].

Estrogen up-regulates COX-1, but also up-regulates PGI_2 synthase expression, thus directing prostanoid balance toward increased PGI_2 production [172]. Testosterone has also been reported to increase PGI_2 production through COX-2 up-regulation in diabetic rats [173].

Sex hormones and vascular remodeling

Since the role of sex hormones on vascular functions has become a theme of great interest in the last decades and since most arterial diseases are related to vascular structure alterations, the effect of sex hormones on vascular remodeling has also been investigated. However, studies concerning sex hormones and vascular remodeling have mainly focused on hypertrophic remodeling, thus addressing the role of sex steroids on proliferation cascades and growth factors. The majority of these studies show that both male and female sex hormones play antiproliferative and anti-inflammatory roles, thus inhibiting the hypertrophic remodeling related to atherosclerotic lesions. Estrogens attenuate atherosclerosis, inhibits oxidative stress, and attenuate vascular remodeling via inhibition of proliferation signalling pathways [174]. Progesterone also attenuates vascular hypertrophic remodeling [175]. Regarding male sex hormones, low serum

levels of testosterone are inversely related to the severity of coronary atherosclerosis [176] carotid intima-media thickening [177, 178] and arterial stiffness [179]. Testosterone ameliorates the expression of inflammation factors, improves lipid profile in hypogonadal men [180], and inhibits vascular calcification [181]. Thus, unlike estrogens, and although there are many studies showing a preventive role of androgens on hypertrophic remodeling, the mechanisms underlying this protection have not been elucidated yet. Androgens have been studied to a lesser extent than estrogens and indeed there are some contradictory results regarding effect of androgens on vascular media thickening, as in some studies the effect was beneficial while in others it was detrimental [70, 182].

Regarding eutrophic remodeling, less evidence can be found on the beneficial effects of sex hormones. Some studies have shown increased arterial stiffening associated with low serum levels of female sex steroids [183], and they clearly demonstrated that sex hormones themselves were able to reverse this situation when serum levels were restored [184]. They also showed that androgens did not contribute to reduce arterial stiffening in males as compared to estrogens in females. Nevertheless, this study does not determine the specific nature of the remodeling, as they only measured arterial stiffness but not vessel diameters. Other authors have addressed the relationship between arterial remodeling, intimal calcification and arterial stiffness, showing that testosterone inhibits vascular calcification [181]. These data also point to the idea that sex hormones may provide vascular protection against pathologic vascular remodeling.

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AIMS AND OUTLINE

The main focus of this thesis was to investigate the role of endogenous male sex hormones on the two mechanisms controlling peripheral vascular resistances, which are vascular tone and vascular structure.

Regarding vascular tone, the studies presented deal with the neural control of vascular tone, analyzing the role of β-adrenoceptors and the participation of some prostanoids. Previous studies have reported that orchidectomy in male rats decreases the vasoconstrictor response to NA, though vasoconstriction induced by EFS and neuronal NO release are not modified. Chapters 2 and 3 of this thesis investigate further the effect of androgens on the processes driving the contractile response to EFS. Specifically, Chapter 2 analyzes possible differences in the EFS-induced NA release between control and orchidectomized rats, and since NA and neuronal NO release can be modulated by β-adrenoceptors, and female sex hormones can influence β-adrenoceptor function, the sensitivity and participation of β-adrenoceptors in NA and NO release in segments from both control and orchidectomized rats were also analyzed. As NA-release was found not to be modified by orchidectomy, the next study, described in Chapter 3, analyzed the participation of TXA_2 in the vasoconstrictor response to EFS in both groups of rats, and the role of TXA_2 on both the release and response to NA and NO. This study also describes an interaction between TXA_2 PGI_2 and NO release in arteries from orchidectomized animals.

Regarding vascular structure, previous studies have shown alterations in the structure of arteries from orchidectomized rats. Hence, the study presented in Chapter 4, aimed to determine the effect of sex hormones on vascular remodeling. It analyzes the possible preventive effect of testosterone on the inward remodeling induced by chronic vasoconstriction in small mesenteric vessels from male rats, as well as the role of TG2 in this effect.

2

Orchidectomy increases β-adrenoceptor activation-mediated neuronal nitric oxide and noradrenaline release in rat mesenteric artery.

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ABSTRACT

Background/Aims. A previous study has demonstrated that endogenous male sex hormones do not alter neuronal nitric oxide (NO) release in rat mesenteric artery. However, the regulatory role of endogenous male sex hormones on noradrenaline (NA) release in rat mesenteric artery is not known. The present study was designed to analyse whether endogenous male sex hormones influence the NA release induced by electrical field stimulation (EFS), as well as the possible modification in NA and neuronal NO release by presynaptic β-adrenoceptor activation.

Methods. For this purpose, mesenteric artery from control and orchidectomized male Sprague-Dawley rats were used. Basal and EFS-induced neuronal NO and noradrenaline release, as well as the contractile effect induced by EFS, was measured.

Results. Basal and EFS-induced neuronal NO and NA release were similar in arteries from control and orchidectomized rats. The β-adrenoceptor agonist clenbuterol did not modify EFS-induced neuronal NO and NA release in arteries from control rats. On the contrary, in arteries from orchidectomized animals, clenbuterol increased both neuronal NO and NA release, this increase was prevented by incubation with the β-adrenoceptor antagonist propranolol. However, the contractile response elicited by EFS was not modified by clenbuterol in either group of rats.

Conclusions. These results show that orchidectomy does not alter the EFS-induced NA release. What is more, activation of presynaptic β-adrenoceptors does not modify EFS-induced NA and neuronal NO release in arteries from control rats although it increases the release of both neurotransmitters in arteries from orchidectomized rats. Despite these modifications, the EFS-induced contractile response is preserved in arteries from orchidectomized rats.

INTRODUCTION

Vascular tone is regulated by several mechanisms in which, depending on the type of the vessel, innervation plays a more or less important role [1-3]. The perivascular sympathetic innervation of rat mesenteric arteries releases noradrenaline (NA) when electrically stimulated [4]. In its turn, the released NA can either induce a contractile response via α-adrenoceptor activation [5, 6] or it can activate β-adrenoceptors that relax the blood vessels [1]. It has also been demonstrated that electrical field stimulation (EFS) induces nitric oxide (NO) release from nitrergic nerves in rat mesenteric arteries [5-8], thus producing relaxation by stimulating soluble guanylate cyclase and increasing the intracellular levels of cGMP in the smooth muscle cells of the arterial wall [9, 10].

The existence of β-adrenoceptors in different vascular wall cell types has been reported. Presynaptic β-adrenoceptors and their role in facilitating NA [11, 12] and neuronal NO [6, 7] release from adrenergic and nitrergic innervations, respectively, in rat mesenteric arteries have been demonstrated. Postsynaptic β-adrenoceptors induce a smooth muscle vasodilator response [1, 13] as well as endothelial NO release [14].

The role of male sex hormones in modulating vascular wall structure and function is the object of recent studies [8, 15]. For many years androgens have been associated with an increased risk of cardiovascular disease, but recent studies have reported that androgens have some protective effects in males [16-19] in which endothelial NO is involved. In this sense, we have described, in rat mesenteric artery, that orchidectomy did not modify endothelial NO release, while it decreased the response induced by acetylcholine [20]. Likewise, we have already studied the role of endogenous male sex hormones in the release of the vasodilator neurotransmitter NO, in the same artery, and we observed that this neuronal NO release was not modified by endogenous male sex hormones [8]. However, little information is available on the specific effect of endogenous male sex hormones in the release of the vasoconstrictor neurotransmitter NA in the peripheral nervous system. Studies on this issue, mostly performed in the central nervous system, have produced contradictory results; decreased [21], increased [22] and unmodified [23] NA release have been reported in orchidectomized animals.

In view of these observations, and taking into account female sex hormone modulation of postsynaptic β-adrenoceptor function in rat mesenteric arteries [13], it would be possible to speculate that male sex hormones could influence presynaptic β-adrenoceptor function, thereby modulating NA and neuronal NO release through β-adrenoceptor activation.

Therefore, the present work analyses whether endogenous male sex hormones influence the NA release induced by EFS in rat mesenteric artery, as well as the possible modification in the NA and neuronal NO release modulated by presynaptic β-adrenoceptors activation.

MATERIALS AND METHODS

Animal housing and protocols

Male Sprague-Dawley rats (6 months old) were used. These were divided into two groups: control and orchidectomized rats. All animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) in accordance with directives 609/86 CEE and R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain. Male sex hormone deprivation was induced by gonadectomy at 7 weeks of age, and 4 months later the animals were sacrificed. The observation of seminal vesicle atrophy confirmed successful surgery. Rats were sacrificed by CO_2 inhalation; the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS, in mM: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03) at 4 ºC. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the USA National Institutes of Health (NIH publication No. 85.23 revised 1985).

Tritium release experiments

Segments of rat mesenteric arteries of 4 mm in length were set up in a nylon net and immersed for 30 min in 10 ml of KHS at 37 ºC continuously gassed with 95% O_2 - 5% CO_2 (stabilisation period). Thereafter, the segments were incubated for 60 min in 1 ml of bubbled KHS at 37 ºC containing (+)-[^{3}H]NA (0.33 µM, 10 µCi/ml, specific activity 10 Ci/mmol). The arteries were then transferred to a superfusion chamber with two parallel platinum electrodes, 0.5 cm apart, connected to a stimulator (Grass, model S44) for EFS. The arteries were superfused at a rate of 2ml/min with oxygenated KHS at 37 ºC for 100 min, during which time the steady-state level of basal tritium efflux was reached. At this time, two samples were collected with an interval of 30 s to determine basal tritium release. Then, an electrical stimulation period of 60 s (S_1; 200 mA, 0.3 ms, 4 Hz) was applied to the arteries while the superfusate was being collected at 30 s intervals. After the S_1 samples, six more samples were also collected, at 30 s intervals, to determine the recovery basal level of the tritium efflux.

At 45 min after the S_1 period, a second stimulation period (S_2) of equal characteristics was performed. In some set of experiments, either clenbuterol or

propranolol, the respective β-adrenoceptor agonist or antagonist used to interfere with tritium release, were administered 30 min before S_2.

Ready-Protein solution (Beckman) was then added to the vials and the radioactivity was measured in a scintillation counter (Beckman LS 5000 TD). Cocaine (10 µM) and normetanephrine (10 µM) were added to the superfusion fluid after the incubation period, and maintained throughout the experiment to block neuronal and extraneuronal uptake of NA respectively.

The stimulation-induced ^3H release was calculated by subtracting basal tritium release from that evoked by EFS. Thereafter, the ratios of the net tritium release between S_2/S_1 were calculated to eliminate differences among arteries. The actions of the drugs on the evoked release were expressed as their effects on these ratios. The amount of radioactivity released was expressed in dpm/mg.

Nitric Oxide release

Endothelium-denuded mesenteric arteries from control and orchidectomized rats were subjected to a resting tension of 0.5 g as indicated for the reactivity experiments. After an equilibration period of 60 min, arteries were incubated with the fluorescent probe 4,5-diaminofluorescein (DAF-2, 0.5 µM) for 45 min. Then, the medium was collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer instruments, FL WINLAB Software) with excitation wavelength set at 495 nm and emission wavelength at 515 nm. Validation of this method has been studied by comparing the results obtained with DAF with those obtained by nitrites measurement (Martín et al., 2005).

The interference of clenbuterol (1 µM), propranolol (1 µM) or clenbuterol plus propranolol on NO release was studied by incubating the arteries with these drugs 30 min before collecting medium to determine basal NO release, or 30 min prior to the EFS application for determination of EFS-induced NO release.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank measures were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of the constitutive NOS inhibitor, N_ω-nitro-L-arginine

methyl ester (L-NAME, 0.1 mM) to ensure the specificity of the method. The amount of NO released was expressed as arbitrary units/mg tissue.

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere [24]. Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, Mass., USA); this was connected in turn to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply adequate current strength. Segments were suspended in an organ bath containing 5 ml of KHS at 37^{o}C continuously bubbled with a 95% O_2-5%CO_2 mixture (pH of 7.4). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g which was readjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mM KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mM KCl. After a washout period, the absence of vascular endothelium was tested by the inability of 10 µM acetylcholine (ACh) to relax segments precontracted with 1 µM noradrenaline (NA).

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) and concentration-response curves to NA (10 nM-10 µM) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1-16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitisation between consecutive curves. Three successive frequency-response curves separated by 1h intervals produced similar contractile response.

To determine the effect of clenbuterol on the response induced by EFS, this drug was added to the bath 30 min before the second frequency-response stimulation.

The ability of clenbuterol to induce relaxation was analysed in NA-precontracted segments from both groups.

The possible effect of clenbuterol on basal tone and on the vasodilator effect of sodium nitroprusside (SNP) was also examined in segments from both rat groups.

Drugs

L-NA hydrochloride, ACh chloride, L-NAME hydrochloride, sodium nitroprusside, clenbuterol hydrochloride, propranolol hydrochloride, DAF-2 (Sigma-Aldrich; Spain); and (+)-[^{3}H]NA hydrochloride from New England Nuclear, Boston, MA, USA. Stock solutions (10 mM) of drugs were made in distilled water, except for NA which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at –20 ºC and appropriate dilutions were made in KHS on the day of the experiment.

Data Analysis

The responses elicited by EFS, KCl, or NA were expressed in mg for comparison between control and orchidectomized male rats. The relaxation induced by SNP or clenbuterol was expressed as a percentage of the initial contraction elicited by NA. Results are given as mean ± SEM. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated-measure analysis of variance (ANOVA). For the experiments of NO and NA release, the statistical analysis was done using a Student's *t* test for unpaired experiments. A *p* value of less than 0.05 was considered significant.

RESULTS

Tritium release experiments

Basal tritium release was similar in arteries from control and orchidectomized rats (control, 95 ± 4.8 dpm/mg; orchidectomized 79.8 ± 5.2 dpm/mg, n= 14; $p > 0.05$). Electrical stimulation induced tritium release; the release obtained in S_2 (control, 333.5 ±31.05 dpm/mg; orchidectomized 353.4 ± 20.14 dpm/mg, n = 4; $p > 0.05$) was similar to that found in S_1 (control, 370.8 ± 28.7 dpm/mg; orchidectomized, 338.1 ± 18.9 dpm/mg, n = 4; $p > 0.05$). The S_2/S_1 ratio for segments from control animals was not significantly different from the one in orchidectomized rats (control, 0.89 ± 0.1; orchidectomized, 1.04 ± 0.09; $p > 0.05$). Tritium release was markedly reduced by 0.1 µM tetrodotoxin (S_2/S_1, 0.04 ± 0.01; n=4, $p < 0.001$) in both groups.

In segments from control rats, neither clenbuterol (1 µM, the β-adrenoceptor agonist) nor propranolol (1 µM, the β-adrenocpetor antagonist) modified the stimulated tritium release (Fig. 1a). In segments from orchidectomized rats, the presence of clenbuterol increased the tritium release induced by EFS, and this increase was blocked by prior incubation with propranolol (Fig. 1b)

Basal tritium release was not affected by clenbuterol or propranolol preincubation in control or orchidectomized rats (data not shown).

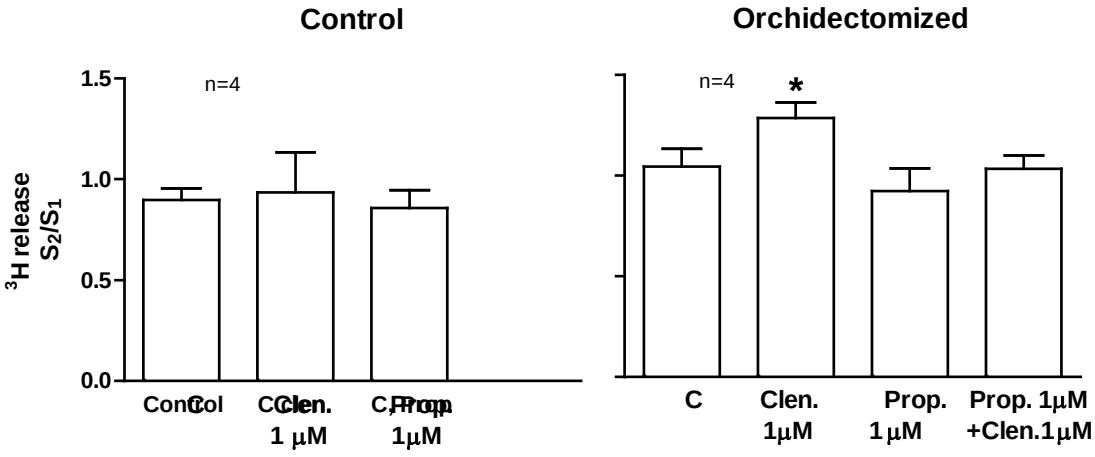

Fig. 1. Effect of clenbuterol (Clen.) or propranolol (Prop.) on the electrically stimulated tritium release in mesenteric artery segments from control rats. Effect of Clen., Prop. or Prop. + Clen. on the electrically stimulated tritium release in mesenteric artery segments from orchidectomized rats. Results (means ± SEM.) are expressed as the ratio S_2/S_1. n = Number of animals. * $p < 0.05$ vs control (C); + $p<0.05$ vs Clen.

Nitric Oxide release

Basal NO release was similar in mesenteric arteries from both control and orchidectomized rats (ANOVA, $p > 0.05$). EFS induced a similar increase of NO release in arteries from both control and orchidectomized rats (ANOVA, $p > 0.05$). In segments from control rats, preincubation with either clenbuterol or propranolol did not modify the basal or the stimulated NO release (Fig. 2a). In contrast, in segments from orchidectomized rats, preincubation with clenbuterol enhanced basal and electrically-stimulated NO release (Fig. 2b). The facilitatory effect induced by clenbuterol was not reversed by preincubation with propranolol (1 µM), but, when the propranolol concentration was increased to 10 µM, the facilitatory effect was blocked (Fig. 2b).

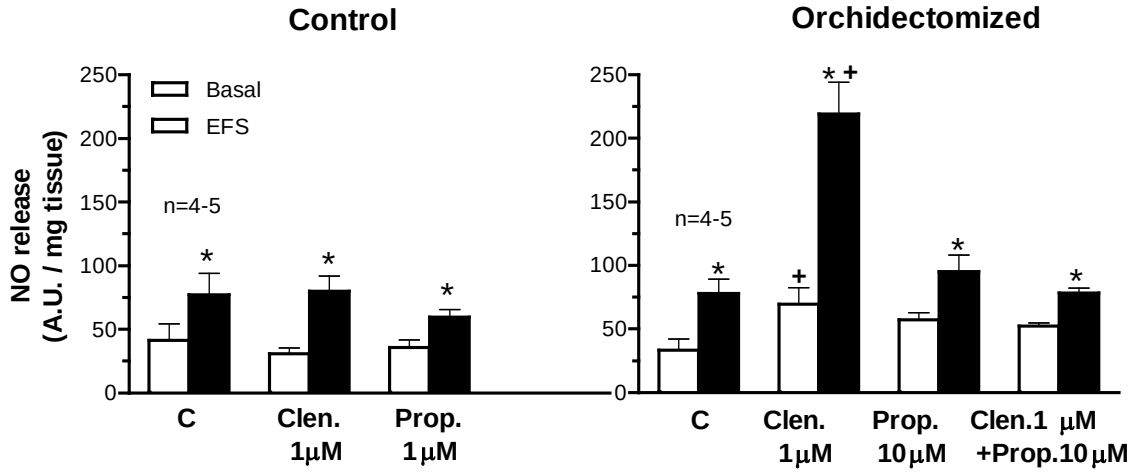

Fig. 2. Effect of clenbuterol (Clen.) or propranolol (Prop.) on the basal- and EFS-induced NO release in mesenteric artery segments from control rats. Effect of Clen., Prop. or Prop. + Clen. on the basal- and EFS-induced NO release in mesenteric artery segments from orchidectomized rats. Results (means ± SEM.) are expressed as arbitrary units. n = Number of animals. * $p < 0.05$ vs the respective basal NO release; + $p<0.05$ vs the basal- and EFS-induced NO release in absence of any drug (C).

Vascular reactivity

The contractile responses induced by KCl or exogenous NA were diminished in arteries from orchidectomized rats. However, the contractile responses induced by EFS were similar in both groups of rats; they were practically abolished by tetrodotoxin (0.1 µM), the blocker for nerve impulse propagation, and markedly

reduced by phentolamine (1 µM), the antagonist of α-adrenoceptors, in segments from both groups, as we have previously reported [8].

In segments from control and orchidectomized rats, the presence of 1 µM clenbuterol did not modify the contractile response to EFS (Fig. 3a, b).

The contractile tone obtained with NA was 1002 ± 72 mg in arteries from control rats and 748 ± 67 mg in arteries from orchidectomized rats, n= 7-10. In these precontracted segments, clenbuterol (10 nM-1 µM) induced a very slight concentration-dependent relaxation, and this relaxation was similar in segments from both control and orchidectomized rats (1 µM induced 7.3 ± 2.2 and 6.5 ± 2.4 % of inhibition of the previous tone with NA, respectively).

Clenbuterol preincubation did not modify the vasodilator response induced by SNP (0.1 nM- 10 µM) in arteries precontracted with NA from either group (Fig. 4).

None of these drugs affected the basal tone or the concentration-dependent contraction curves to NA (data not shown).

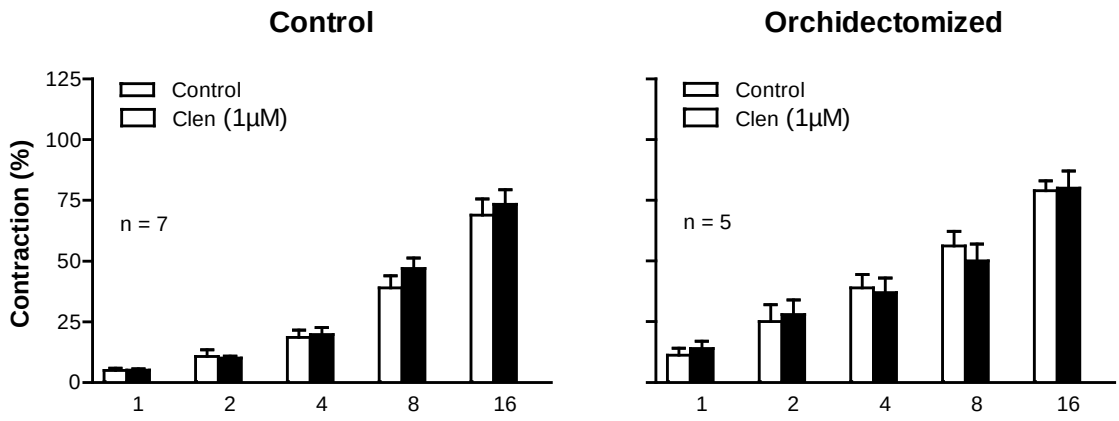

Fig. 3. Effect of clenbuterol (Clen) on the frequency-response curve in mesenteric artery segments from control and orchidectomized rats. Results (means ± SEM.) are expressed as a percentage of tone induced by 75 mM KCl. n = Number of animals.

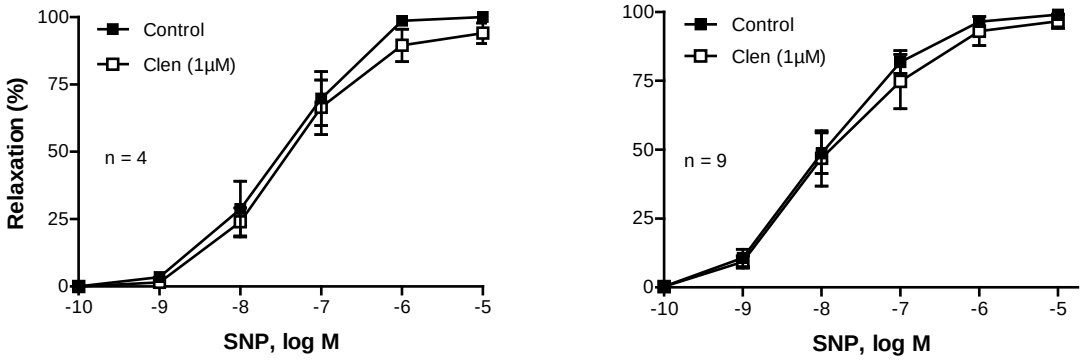

Fig. 4. Effect of clenbuterol (Clen) on the concentration-response curve to sodium nitroprusside (SNP) in mesenteric artery segments from control and orchidectomized rats. Results (means ± SEM.) are expressed as a percentage of a previous tone with noradrenaline. n = Number of animals.

DISCUSSION

The results of the present study demonstrate that endogenous male sex hormones are involved in the regulation of the presynaptic β-adrenoceptor function in rat mesenteric artery. In fact, activation of presynaptic β -adrenoceptors facilitates NA and neuronal NO release in mesenteric arteries from orchidectomized rats, but not in arteries from control rats. In spite the changes produced in arteries from orchidectomized rats, the contractile response induced by EFS remains unaltered.

The balance between opposing vasoconstrictor and vasodilator forces is one of several factors that control vascular tone [6, 25]. Rat mesenteric arteries possess rich sympathetic [4], sensory motor [3] and nitrergic innervations [7, 8], which modulate vasomotor tone. In a previous work we have demonstrated that EFS induced contractile response in endothelium-denuded mesenteric segments from control and orchidectomized male rats; these responses appear to be mediated by NA release from adrenergic nerve terminals and the subsequent activation of α-adrenoceptors in both experimental hormonal conditions, as has been described in other rat strains [5, 26]. We also demonstrated the involvement of neuronal NO in the EFS-induced response, and quantification of the NO release showed that EFS induced similar releases of neuronal NO in both control and orchidectomized rats [8]. Our results indicate the involvement of neuronal NO in the EFS-induced response in rat mesenteric artery, but they do not determine the anatomical identity of the nitrergic perivascular nerves involved in NO release. In this sense, a recent report shows that perivascular capsaicin-sensitive nerves release NO [27], although we observed that sensory innervation was not involved in the vasomotor response to EFS in segments from both control and orchidectomized rats [8].

The fact that the contractile response to exogenous NA was diminished in arteries from orchidectomized rats while the EFS response was similar in both groups of rats, suggested that EFS could modulate neurotransmitter release in mesenteric arteries from orchidectomized rats. Studies on the modulatory effect of male sex hormones in NA release in the central nervous system have contradictory results: increase [22, 28], decrease [21, 29] and no modification [23, 30]. However, the issue of male sex hormones modulating NA release had not been studied in the peripheral nervous system. Therefore, we analysed the influence of endogenous male sex hormones on NA release by studying the possible changes in the tritium overflow induced by EFS

in [^{3}H]-NA preincubated segments from both rat groups. The results show that orchidectomy did not modify the tritium overflow induced by EFS, suggesting that other vasoconstrictor factors were released when the artery was electrically stimulated.

Nevertheless, modulation of NA [12] and neuronal NO [7] release by presynaptic β-adrenoceptor has been reported, although these effects have not been observed in all tissues [6, 11]. Additionally, we know that β-adrenoceptor function can be altered in physiopathological conditions, such as aging or hypertension, in endothelial cells [31], smooth muscle cells [32, 33] and presynaptic endings [34, 35]. Since modulation of β-adrenoceptor function by female sex hormones has been described [13], it is possible to speculate that male sex hormones could also modulate presynaptic β-adrenoceptor function. To investigate this possibility, we studied the effect of the β-adrenoceptor agonist clenbuterol on noradrenergic neurotransmission by assessing its capacity to modulate the tritium overflow induced by EFS. Clenbuterol did not significantly alter the EFS-induced overflow in segments from control rats, confirming that activation of presynaptic β-adrenoceptors does not influence noradrenergic transmission in mesenteric arteries from control animals, as shown previously [6, 36]. However, the activation of presynaptic β-adrenoceptors increased NA release in arteries from orchidectomized rats. The fact that propranolol prevented the facilitatory effect induced by clenbuterol indicates that this effect is mediated by β-adrenoceptor activation, and this activation could result in an increased local peripheral resistance.

Since functional studies have shown that neuronal NO release is modulated by presynaptic β-adrenoceptors in different rat strains [6, 7], we analysed the effect of β-adrenoceptor activation on neuronal NO release by measuring the fluorescence emitted by DAF-2. We observed that, in segments from control rats, clenbuterol and propranolol both failed to modify the basal and EFS-induced neuronal NO release. On the contrary, clenbuterol increased both the basal and the electrically-stimulated NO release in arteries from orchidectomized rats. The fact that the β-adrenoceptor antagonist, propranolol, prevented the clenbuterol-induced NO release indicates that the facilitatory effect is mediated by β-adrenoceptor activation. It is important to note that to reverse the facilitatory effect on neuronal NO release induced by clenbuterol, it was necessary to use a higher concentration of propranolol than the concentration needed to reverse its effect on NA release. This fact could also indicate that the

presynaptic β-adrenoceptors modulating NO release seem to be more numerous and/or have a greater affinity for clenbuterol than those modulating NA release. However, what is of importance is that the activation of β-adrenoceptors increased EFS-induced neurogenic NA and NO only in orchidectomized rats.

The next step was to investigate the functional role of β-adrenoceptor activation in the arterial vasomotor response induced by EFS. We observed that the EFS-induced contractile response was not modified by clenbuterol in arteries from control or orchidectomized rats. These results agree with the fact that clenbuterol does not modify either the neuronal NO or NA release in mesenteric arteries from control animals. This result differs from that presented by Marín and Balfagón [7], where clenbuterol inhibited the contraction induced by EFS. This apparent discrepancy could be due to the different rat strains used in the two studies. However, although the net effect of clenbuterol on EFS-induce contraction in arteries from orchidectomized rats was similar to that produced in arteries from control rats, the mechanisms involved are different. In arteries from orchidectomized rats, the non modification observed in the presence of clenbuterol is due, at least in part, to an increased release of the two opposing vasoconstrictor and vasodilator neurotransmitters: NA and NO respectively, as was demonstrated with the NA and NO release experiments. A possible interaction between β-adrenoceptor activation and the vascular effect of neuronal NO was ruled out since the vasodilator action of sodium nitroprusside was not modified by preincubation with clenbuterol.

Having described a modulatory action on postsynaptic α-adrenoceptors by endogenous male sex hormones [37], we further investigated the possible effect of orchidectomy on postsynaptic β-adrenoceptor function by performing concentration-dependent relaxation curves to clenbuterol. We observed that clenbuterol induced a very modest relaxation in segments precontracted with NA, and that this relaxation was similar in arteries from both rat groups; this suggests that orchidectomy does not affect the function of postsynaptic β-adrenoceptors. On the other hand, the possible interaction between α- and β-adrenoceptors was studied, because increases [38] and decreases [39] in α-adrenoceptor-mediated contraction have both been described in the presence of the β-agonists. We observed that preincubation with clenbuterol did not modify the contraction elicited by exogenous NA in arteries from either control or orchidectomized rats, indicating that activation of β-adrenoceptors

had no effect on the response induced by α-adrenoceptor activation in arteries from either rat groups.

In conclusion, our results show that orchidectomy does not alter EFS-induced NA release. Additionally, activation of presynaptic β-adrenoceptors does not modify NA and neuronal NO release in mesenteric arteries from control rats. However, in arteries from orchidectomized rats, presynaptic β-adrenoceptor activation increases the EFS-induced NA release. This increase in available NA in the absence of male hormones would intensify the vasomotor and trophic action of NA on the vessel wall [40, 41] and thereby, probably affect cardiovascular function adversely. In addition, β-adrenoceptor activation also increases neuronal NO release in these orchidectomized rats, in what may be an attempt to compensate for the vascular actions produced by the NA.

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3

Orchidectomy increases the formation of non-endothelial thromboxane A2 and modulates its role in the electrical field stimulation-induced response in rat mesenteric artery.

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ABSTRACT

The aim of the present study was to analyze whether endogenous male sex hormones influence the release of thromboxane TXA_2 and its role in the electrical field stimulation (EFS)-induced response, as well as the mechanism involved. For this purpose, endothelial-denuded mesenteric arteries from control and orchidectomized male Sprague-Dawley rats were used to measure TXA_2 release; EFS-induced response, nitric oxide (NO), noradrenaline (NA) and prostaglandin (PG) I_2 release were also measured in the presence of the TXA_2 synthesis inhibitor furegrelate. Orchidectomy increased basal and EFS-induced TXA_2 release. Furegrelate decreased the EFS-induced contraction in arteries from control rats, but did not modify it in arteries from orchidectomized rats. The EFS-induced neuronal NO release and vasodilator response was increased by furegrelate in arteries from control rats, but were not modified in arteries from orchidectomized rats. Furegrelate did not modify the EFS-induced NA release or vasoconstrictor response in arteries from either control or orchidectomized rats. The EFS-induced PGI_2 release was not modified by furegrelate in arteries from control rats, but was increased in arteries from orchidectomized rats. The results of the present study show that endogenous male sex hormone deprivation: (i) increases non-endothelial TXA_2 release, and (ii) regulates the effect of endogenous TXA_2 on the EFS-induced response through different mechanisms that, at the least, involve the NO and PGI_2 systems. In arteries from control rats, inhibition of TXA_2 formation decreases the EFS-induced response by increasing neuronal NO release. In arteries from orchidectomized rats, the EFS-induced response is unaltered after the inhibition of TXA_2 formation, by increasing PGI_2 release.

INTRODUCTION

Clinical and epidemiological studies indicate the existence of gender differences in the incidence of cardiovascular disease and have established the existence of vascular protective effects of endogenous estrogens (Teede 2007). On their part, androgens have generally been associated with impaired vascular reactivity; nevertheless, recent studies have reported beneficial properties of androgens in male vascular function (Blanco-Rivero *et al*. 2006*a,b*) similar to the effects of estrogens in women (Alexandersen *et al*. 1999, Ng 2007). In addition, low levels of testosterone have been reported in patients with hypertension (Phillips *et al*. 1993), atherosclerosis (Alexandersen *et al*. 1996) and coronary disease (Wranicz *et al*. 2005).

Vascular tone is regulated by several mechanisms in which, depending on the type of the vessel, innervation plays a more or less important role. This regulation involves the adrenergic, cholinergic, nitrergic, peptidergic and/or sensory innervations (Vanhoutte *et al*. 1981; Marco *et al*. 1985; Kawasaki *et al*, 1988), that are specific to the vascular bed under consideration. Nitric oxide (NO) is an important neurotransmitter in both the central (Bredt *et al*. 1992) and the peripheral (Marín & Balfagón 1998) nervous systems. Electrical field estimulation (EFS) has been shown to induce NO release from nitrergic nerves in rat mesenteric arteries (Ferrer *et al*. 2000, Ferrer & Balfagón 2001, Marín & Balfagón 1998, del Carmen Martín *et al*. 2005), thus producing relaxation by stimulating soluble guanylate cyclase and increasing the intracellular levels of cGMP in the smooth muscle cells of the arterial wall (Holtzmann 1982, Ignarro & Kadowitz 1985). Sex hormones have been described to modulate the release and/or function of the neuronal NO in male (del Carmen Martín *et al*. 2005) and female (Minoves *et al*. 2002) rat mesenteric artery.

Vascular tone is also regulated by prostanoids originated by the arachidonic acid metabolism through the cyclooxygenase (COX) pathway (Henrion *et al*. 1997, Félétou & Vanhoutte 2006, Blanco-Rivero *et al*. 2005). One of the most studied prostanoids is thormboxane A_2 (TXA_2), which has been implicated as a mediator in diseases such as myocardial infarction, hypertension and stroke (FitzGerald *et al*. 1987, Narumiya *et al*. 1999). We previously reported that, in mesenteric artery from comparable rats, endogenous male sex hormones modulate endothelial TXA_2 production, whether in basal conditions or after stimulation with either clonidine (Blanco-Rivero *et al*. 2006*a*) or acetylcholine (Blanco-Rivero *et al*., 2007), without modifying the TXA_2 vasoconstrictor

effect. Additionally, we have also shown that endogenous male sex hormones regulate the functional involvement of endogenous TXA_2 in vascular responses of aorta (Martorell *et al.* 2008) and mesenteric artery (Blanco-Rivero *et al*. 2006*a*, Blanco-Rivero *et al*. 2007).

On the other hand, while NO has been reported to modulate the prostanoid system (Salvemini *et al*. 1996, Laemmel *et al*. 2003), there have been few reports on the action of prostanoids on the NO system (Ferrer *et al*. 2004, Mollace *et al*. 2005). Moreover, regarding the specific action of TXA_2 on the NO system, a decrease in inducible (Yamada *et al*. 2003) as well as endothelial (Miyamoto *et al*. 2007) NO release has been reported. However details about the action of TXA_2 on neuronal NO release in vascular tissue remain unknown.

In light of these considerations, the present study was designed to investigate whether endogenous male sex hormones influence the release of TXA_2 and the role of the latter in the electrical field stimulation (EFS)-induced response, as well as the mechanism involved in this response.

MATERIALS AND METHODS

Animal housing and protocols

Male Sprague-Dawley rats (6 months old) were used. Animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) in accordance with directives 609/86 CEE and R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain. Deprivation of male sex hormones was induced by gonadectomy at 7 weeks of age, and 4 months later the animals were sacrificed. The observation of seminal vesicles atrophy confirmed successful surgery. Rats were weighed and sacrificed by CO_2 inhalation; the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS, in mM: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03) at 4^o C. Endothelium was removed to eliminate the main source of vasoactive substances, including NO. This avoided possible actions on endothelial cells by different drugs that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the USA National Institutes of Health (NIH publication No. 85.23 revised 1985).

Thromboxane A_2, noradrenaline and prostaglandin I_2 (PGI_2) release

The production of TXA_2 and PGI_2 in vivo are typically monitored by measuring their stable metabolites TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively, using a TXB_2 or a 6-keto-$PGF_{1\alpha}$ EIA kit (Cayman Chemical). Noradrenaline was measured using Noradrenaline Research EIA (Labor Diagnostika Nord).

Endothelial-denuded rat mesenteric segments were preincubated for 30 min in 5 ml KHS at 37 ºC, continuously gassed with a 95 % O_2-5% CO_2 mixture (stabilization period). After several 10 min washout periods in a bath containing 400 µl KHS, the medium was collected to measure basal release. Once the chamber was refilled cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied, and the medium was collected to measure EFS-induced release. Each assay was made following the manufacturer's instructions. Results were expressed as pg / mL.mg tissue for TXA_2 and PGI_2 release and as ng / mL.mg tissue for NA release.

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere (Nielsen & Owman 1971). Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, Mass., USA); this was connected in turn to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply appropiate current strength. Segments were suspended in an organ bath containing 5 ml KHS at 37^{o}C continuously bubbled with a 95% O_2-5%CO_2 mixture (pH of 7.4). The segments were subjected to a tension of 0.5 g which was readjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mM KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mM KCl. After a washout period, the absence of vascular endothelium was tested by the inability of 10 µM acetylcholine (ACh) to relax segments precontracted with 1 µM noradrenaline (NA).

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) and concentration-response curves to NA (10 nM-10 µM) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1-16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Three successive frequency-response curves separated by 1h intervals produced similar contractile responses.

To determine the effect of endogenous TXA_2 on the response induced by EFS the TXA_2 synthase inhibitor, furegrelate (1 µM), was added to the bath 30 min before the second frequency-response curve.

To determine the possible effect of endogenous TXA_2 on the NA-induced vasoconstrictor response furegrelate was added to the bath 30 min before performing the NA concentration response curve. The possible effect of furegrelate on the vasodilator effect of NO was also analyzed by performing concentration-response curves to the NO donor sodium nitroprusside (SNP) in 30 min furegrelate preincubated arteries.

Nitric Oxide release

Endothelium-denuded mesenteric arteries from control and orchidectomized rats were subjected to a resting tension of 0.5 g as indicated for the reactivity experiments. After an equilibration period of 60 min, arteries were incubated with the fluorescent probe 4,5-diaminofluorescein (DAF-2, 0.5 µM) for 45 min. Then the medium was collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer instruments, FL WINLAB Software) with excitation wavelength set at 495 nm and emission wavelength at 515 nm. This method has been validated by comparing the results obtained with DAF with those obtained by nitrites measurement (del Carmen Martín *et al*. 2005).

The interference of endogenous TXA_2 on NO release was studied by incubating the arteries with the TXA_2 synthase inhibitor furegrelate (1 µM) 30 min before collecting medium.

Each data was calculated by subtracting the blank measures from the corresponding NO release obtained. Blank measures were collected in the same way from segment-free medium in order to subtract background emission. The specificity of the method has already been demonstrated (del Carmen Martín *et al*. 2005, Blanco-Rivero *et al*. 2006*c*). The amount of NO released was expressed as arbitrary units/mg tissue.

Drugs

L-NA hydrochloride, ACh chloride, L-NAME hydrochloride, sodium nitroprusside, DAF-2 (Sigma-Aldrich; Spain); furegrelate (Cayman chemical, Europe). Stock solutions (10 mM) of drugs were made in distilled water, except for NA which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at -20 ºC and appropriate dilutions were made in KHS on the day of the experiment.

Data Analysis

The responses elicited by EFS or NA were expressed as a percentage of the contraction induced by 75 mM KCl. The relaxation induced by SNP was expressed as a percentage of the initial contraction elicited by 1µM NA. Results are given as mean ± SEM. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated-

measure analysis of variance (ANOVA). For the experiments on TXA_2, NO, NA and PGI_2 release, the statistical analysis was done using a Student's *t* test for unpaired experiments. A *p* value of less than 0.05 was considered significant.

RESULTS

Body weight

Orchidectomy slightly decreased rat body weight (control, 469.7 ± 8.4 g; orchidectomized, 428 ± 5.4 g; n= 10; $p < 0.05$), but this did not affect the size of mesenteric artery.

Thromboxane A_2 release

Orchidectomy increased basal TXA_2 release. The EFS-induced TXA_2 release was greater in arteries from orchidectomized than control male rats (Fig. 1).

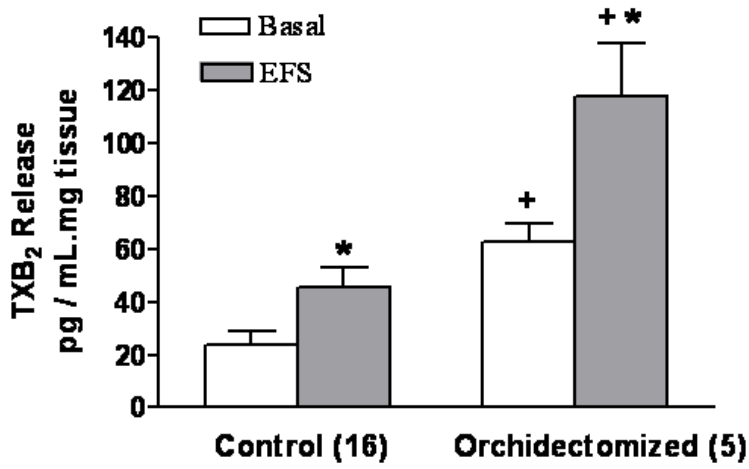

Figure 1. Basal and EFS-induced thromboxane A_2 (TXA_2) release in mesenteric denuded segments from control (A) and orchidectomized (B) rats. Results (mean ± S.E.M) are expressed as pg/mL.mg tissue. Number of animals is indicated in parenthesis. *P<0.05 compared with basal TXA_2 release. ^{+}P<0.05 compared with the respective control.

Vascular reactivity

Preincubation with the TXA_2 synthase inhibitor furegrelate (1 µM) did not modify basal tone in arteries from either rat group; furegrelate decreased the EFS-induced contraction in arteries from control male rats (Fig. 2a), but did not modify it in arteries from orchidectomized rats (Fig. 2b).

Preincubation with furegrelate did not modify the NA-induced contraction in arteries from control (Fig. 3a) or orchidectomized rats (Fig. 3b).

Preincubation with furegrelate increased the SNP-induced relaxation in arteries from control male rats (Fig.4a), but did not affect that induced in arteries from orchidectomized rats (Fig.4b).

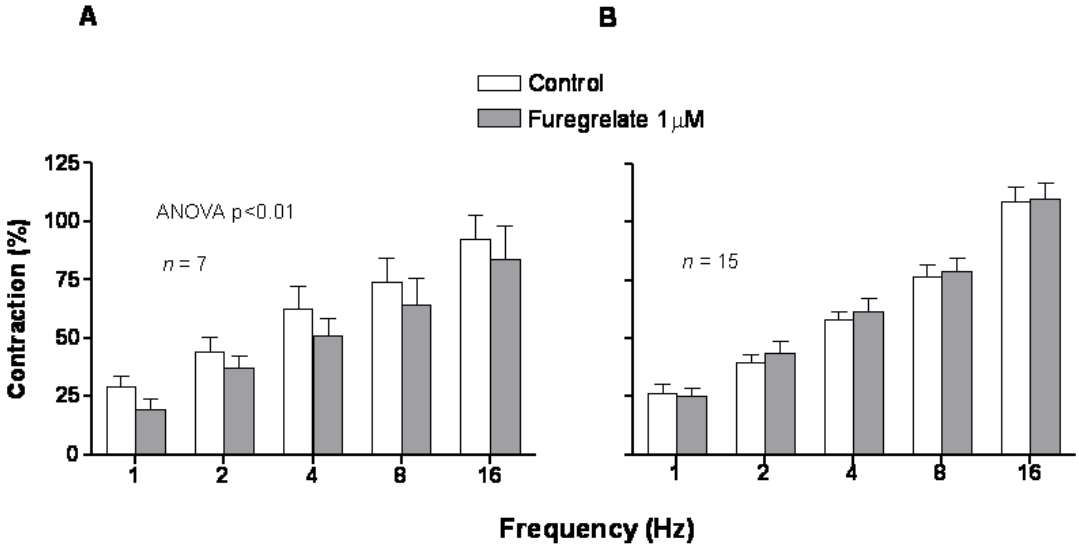

Figure 2. Effect of furegrelate on the frequency–response curve in mesenteric artery segments from control (A) and orchidectomized (B) rats. Results (mean ± S.E.M) are expressed as a percentage of the tone induced by 75 mM KCl. *n* = number of animals.

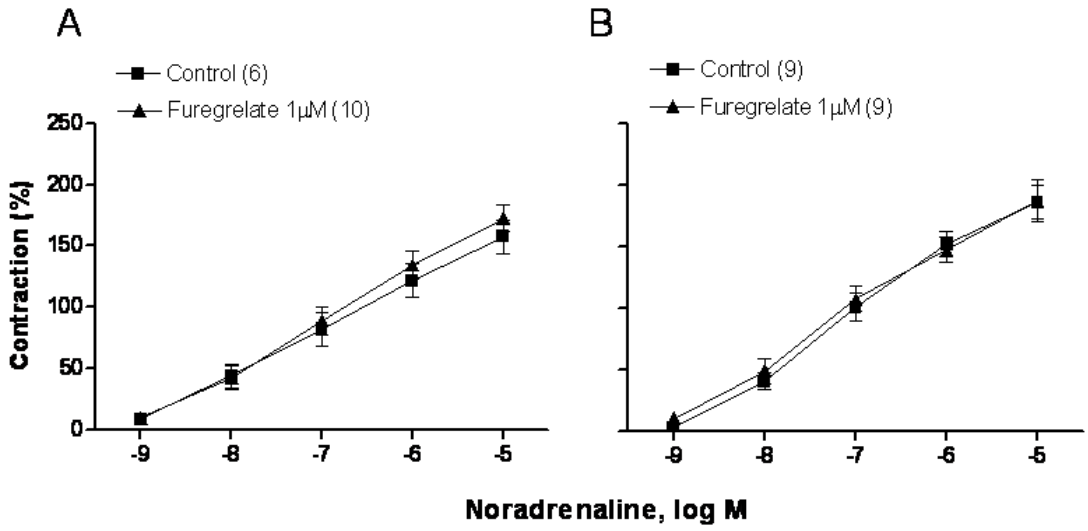

Figure 3. Effect of furegrelate on the concentration-response curves to noradrenaline in mesenteric artery segments from control (A) and orchidectomized (B) rats. Results (mean ± S.E.M.) are as a percentage of the tone induced by 75 mM KCl. Number of animals is indicated in parenthesis.

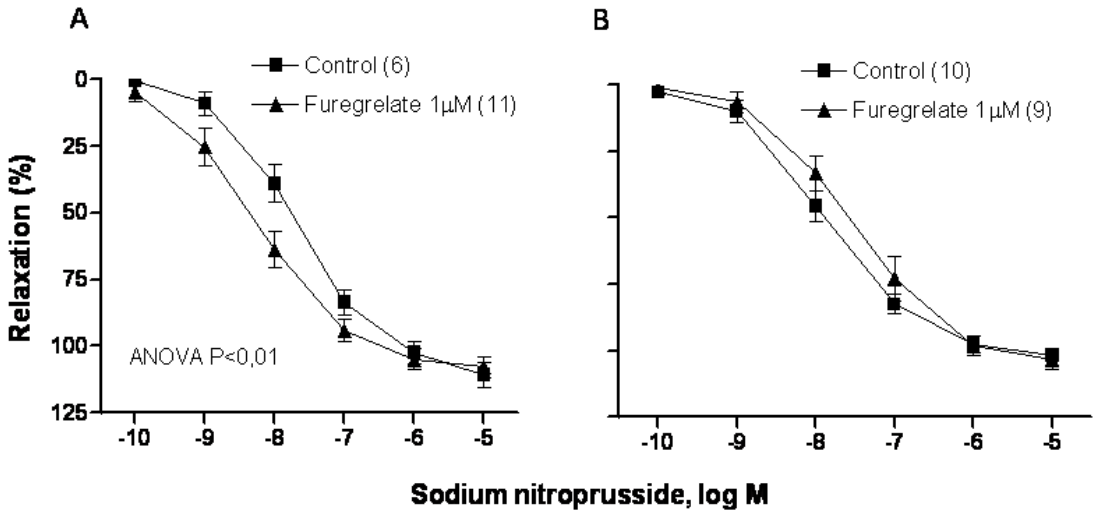

Figure 4. Effect of furegrelate on the concentration-dependent curves to sodium nitroprusside in mesenteric artery segments from control (A) and orchidectomized (B) rats. Results (mean ± S.E.M.) are expressed as a percentage of inhibition of contraction induced by 1 µM NA. Number of animals is indicated in parenthesis.

Nitric Oxide release

Basal and EFS-induced NO release was similar in mesenteric arteries from both control and orchidectomized male rats (ANOVA, $p > 0.05$). In segments from control male rats, preincubation with the TXA_2 synthase inhibitor furegrelate did not modify the basal NO release, but did increase EFS-induced NO release (Fig. 5a). In contrast, in segments from orchidectomized rats, preincubation with furegrelate did not affect basal or EFS-induced NO release (Fig. 5b); similar results were obtained when the furegrelate concentration was increased to 10 µM (Fig. 5b).

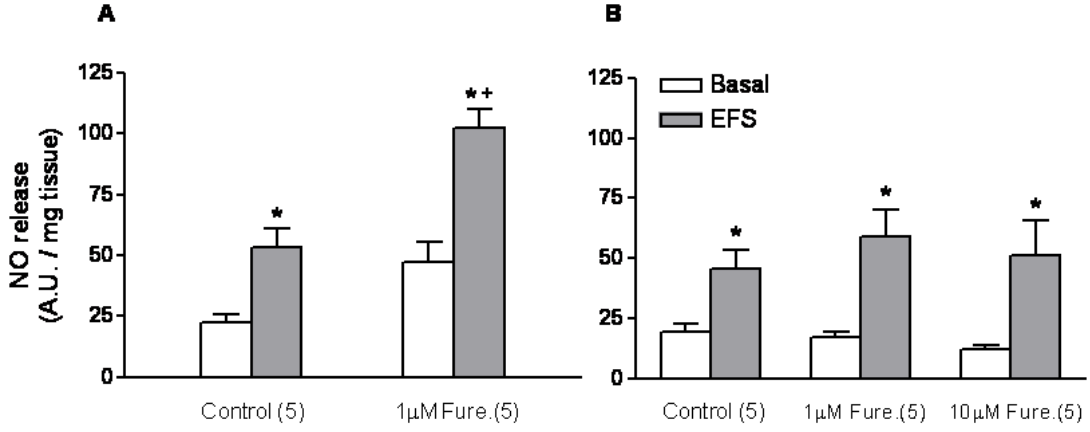

Figure 5. Effect of furegrelate (Fure.) on the basal and EFS-induced NO release in mesenteric denuded segments from control (A) and orchidectomized (B) rats. Results (means ± S.E.M) are expressed as arbitrary units/mg tissue. Number of animals is indicated in parenthesis. * *P*<0.05 compared with basal TXA_2 release. $^{+}$ *P*<0.05 compared with the respective control.

Noradrenaline release

Basal and EFS-induced NA release was similar in mesenteric arteries from both control and orchidectomized male rats (ANOVA, $p > 0.05$), as already reported using the tritium release method for measuring NA release (Blanco-Rivero *et al*. 2006*c*). The presence of furegrelate did not modify either the basal or the EFS-induced NA release in arteries from control (Fig. 6a) or orchidectomized (Fig. 6b) rats.

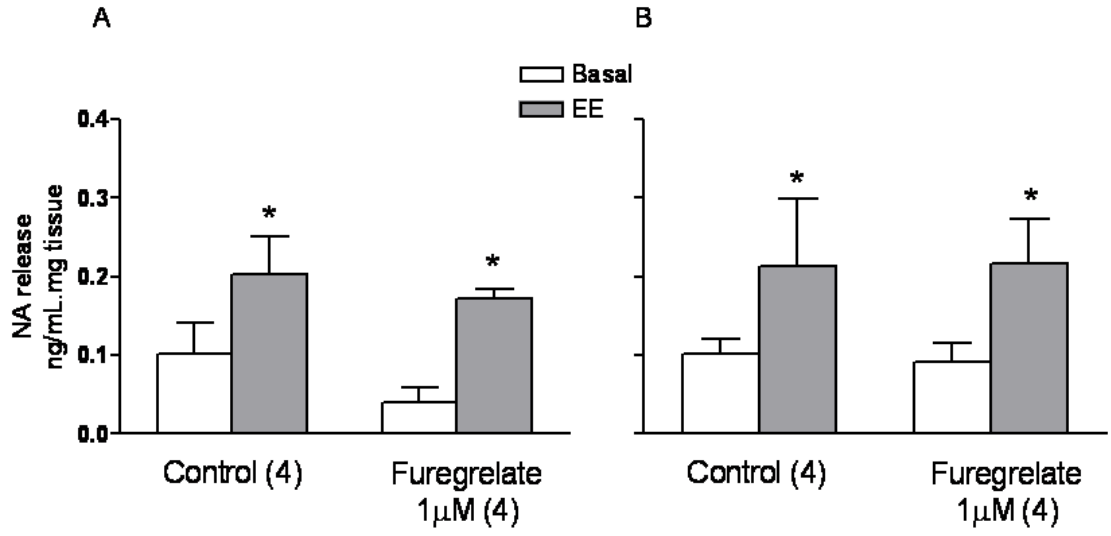

Figure 6. Effect of furegrelate on the basal and EFS-induced noradrenaline (NA) release in mesenteric denuded segments from control (A) and orchidectomized (B) rats. Results (means ± S.E.M) are expressed as ng /mL.mg tissue. Number of animals is indicated in parenthesis. * *P*<0.05 compared with basal NA release. $^{+}$ *P*<0.05 compared with the respective control.

PGI_2 release

Basal and EFS-induced PGI_2 release was similar in mesenteric arteries from both control and orchidectomized male rats (ANOVA, $p > 0.05$). Furegrelate did not modify this release in arteries from control rats (Fig. 7a) but increased it in arteries from orchidectomized rats (Fig. 7b).

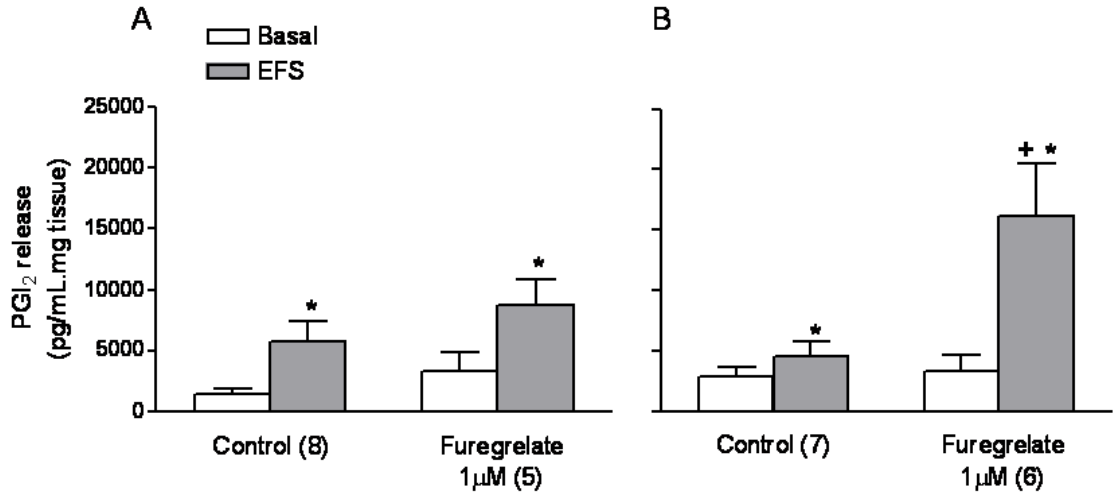

Figure 7. Effect of furegrelate on the basal and EFS-induced PGI_2 release in mesenteric denuded segments from control (A) and orchidectomized (B) rats. Results (means ± S.E.M) are expressed as pg / mL.mg tissue. Number of animals is indicated in parenthesis. * $P<0.05$ compared with basal PGI_2 release. $^{+}$ $P<0.05$ compared with the respective control.

DISCUSSION

Recent studies have reported beneficial effects of androgens in cardiovascular function in males through different mechanisms that involve NO and prostanoids (Jones *et al*. 2004, Martorell *et al.* 2008). The activation of androgen receptors, expressed in both endothelial (Yu *et al*. 2007) and smooth muscle (Ma *et al*. 2005) cells, regulates cell-signalling pathways. However, the vascular effects of male sex hormones have also been reported to be independent of androgen receptor activation (Liu *et al*. 2007) and to interact with intracellular calcium regulatory mechanisms, as reported for other sex steroids hormones (Zhang *et al*. 2006).

The role of NO and prostanoids in regulating vascular tone is well-established (Henrion *et al*. 1997, Ferrer & Osol 1998, Busse & Fleming 2003), and interaction between these two systems has been described, particularly that of NO acting on prostanoids release (Laemmel *et al*. 2003, Mollace 2005), however reports describing action of prostanoids on the NO system are scarce (Ferrer *et al*. 2004). TXA_2 is one of the most important vasoconstrictor prostanoids with stimulatory action on proliferation or hypertrophy of vascular smooth muscle cells (Hanasaki *et al*. 1990), and is implicated as a mediator in diseases such as myocardial infarction, hypertension and stroke (FitzGerald *et al*. 1987, Narumiya *et al*. 1999). We previously reported that endothelial TXA_2 release in mesenteric artery (Blanco-Rivero *et al*. 2006*a*, Blanco-Rivero *et al*. 2007) and aorta (Martorell *et al*. 2008) was increased in arteries from orchidectomized rats. The fact that the levels of testosterone dramatically decreased in orchidectomized rats (Martorell *et al*. 2008), seems to indicate that the vascular effects observed are testosterone-dependent. However, the involvement of hormones and/or gonadal factors other than testosterone can not be ruled out. Thus, vascular endothelial growth factor, basic fibroblast growth factor, transforming growth factor b or hyalurodinase with gonadal origin (Lissbrant *et al*. 2003), could all play an important role in vascular function (Rahmanian & Heldin 2002).

Therefore, we now studied the possible modification of EFS-induced TXA_2 release by endogenous male sex hormones. We found that orchidectomy increased the basal TXA_2 release, as previously reported in endothelium intact mesenteric arteries from comparable animals (Blanco-Rivero *et al*. 2006*a*, Blanco-Rivero *et al*. 2007); it is important to mention that basal release in arteries without endothelium was lower than

in arteries with intact endothelium, confirming endothelial and smooth muscle cells as sources of TXA_2 production.

We have previously demonstrated that EFS induced similar contractile responses in mesenteric arteries from control and orchidectomized rats, responses that appear to be mediated by NA release from adrenergic nerve terminals and the subsequent activation of α-adrenoceptors (del Carmen Martín *et al.* 2005); in addition, we found that the contractile response to exogenous NA was decreased by orchidectomy (del Carmen Martín *et al*. 2005), suggesting that EFS could increase noradrenaline release in arteries from orchidectomized rats; however we later demonstrated that the EFS-induced NA release was not modified by orchidectomy (Blanco-Rivero. 2006c), which indicates that other vasoconstrictor factors could be released when the artery was electrically stimulated. Since EFS-induced release of TXA_2 has been demonstrated in hypertensive rats (Aras-López *et al*. 2007), we analyzed EFS-induced TXA_2 release in normotensive rats, as well as the possible role of endogenous male sex hormones on that release. We observed that EFS induced a greater TXA_2 formation in arteries from orchidectomized than control rats, which is in line with reports showing increased TXA_2 release after activation of different receptors (Blanco-Rivero *et al*. 2006*a*, Blanco-Rivero *et al*. 2007). This result also indicates that TXA_2 could be the contractile factor that is released when the artery is electrically stimulated, as previously suggested (Blanco-Rivero *et al*. 2006*c*). Increased TXA_2 release would explain the non modification of the EFS-induced response in arteries from control and orchidectomized rats, in spite of the fact that the NA response was diminished in arteries from the latter animals.

The next step was to analyze the function of endogenous TXA_2 in the EFS-induced response, as well as the dependence on male sex hormones. Preincubation with furegrelate did not modify the basal tone in arteries from control or orchidectomized rats, indicating that endogenous TXA_2 does not have a direct effect on vascular tone regulation in basal conditions. We showed that furegrelate decreased the EFS-induced response in arteries from control rats, but did not modify it in arteries from orchidectomized rats, indicating that the effect of endogenous TXA_2 on the EFS response is under male sex hormone regulation.

It is widely reported that mesenteric arteries possess nitrergic (Marín & Balfagón 1998, del Carmen Martín *et al*. 2005), sympathetic (Li & Duckles 1992) and sensory (Kawasaki *et al*. 1988) innervations, which modulate vasomotor tone; therefore, the EFS-induced contraction is the result of a balance between opposing vasoconstrictor

and vasodilator factors (Ferrer & Balfagón 2001, Vanhoutte 1996). Since, in our experimental conditions we have demonstrated that sensory innervation does not modulate the vasomotor response to EFS (del Carmen Martín *et al*. 2005), we studied whether the differences in the EFS-induced contractions observed in the presence of furegrelate were due to alterations in nitrergic and adrenergic innervations.

Since there is a lack of studies analyzing the effect of TXA_2 on neuronal NO release and since we previously reported that endogenous prostanoids different from TXA_2, i.e. PGI_2, increased neuronal NO release (Ferrer *et al*. 2004), it is possible to speculate that endogenous TXA_2 could regulate neuronal NO and vasomotor function. Therefore, we studied the effect of the TXA_2 synthesis inhibitor furegrelate, on the release and function of neuronal NO, as well as the dependence on endogenous male sex hormones. We found that in arteries from control rats, furegrelate increased the neuronal NO release, which is in line with reports describing an inhibitory effect of TXA_2 on inducible (Yamada *et al*. 2003) and on endothelial (Miyamoto *et al*. 2007) NO release. The vasodilator response induced by the NO donor, sodium nitroprusside, was also increased by furegrelate, showing that endogenous TXA_2 negatively modulates both the release and the vasodilator effect of neuronal NO. These results could also explain the decreased EFS-induced contraction in the presence of furegrelate, but alterations in the release and function of neurotransmitters other than NO can not be ruled out.

Different modulating effects of TXA_2 on NA release have been reported, including inhibition (Nishihara *et al*. 2000) and non modification (Rump & Schollmeyer 1989). The fact that furegrelate did not modify either NA release or it vasomotor response, as previously reported (Hoang *et al*. 2003, Moldering *et al*. 1998), indicates that endogenous TXA_2 does not alter the function of sympathetic innervation in arteries from control rats.

In contrast, in arteries from orchidectomized rats, furegrelate did not modify the basal and EFS-induced NO release; since TXA_2 formation was greater in arteries from orchidectomized than control rats, we used a higher concentration of furegrelate, and still obtained similar results. In addition, the vasodilator response induced by sodium nitroprusside was not modified by furegrelate. These results show that endogenous TXA_2 does not regulate the release or function of neuronal NO in arteries from orchidectomized rats, in contrast to what occurs in arteries from control rats.

Regarding noradrenergic neurotransmission, we found that furegrelate modified neither NA release nor the vasoconstrictor response induced by exogenous NA, indicating that

the function of the sympathetic innervation is not regulated by endogenous TXA_2 in arteries from orchidectomized rats, as was also observed in arteries from control rats.

Since TXA_2 was higher in arteries from orchidectomized rats, and since that endogenous TXA_2 did not modify either the release or function of NO or NA, the unaltered EFS-induced response observed in the presence of furegrelate could be explained through the release of vasodilator factors that would counterbalance the vasoconstrictor effect of TXA_2.

One of the more plausible candidates would be PGI_2, since cross-talk between TXA_2 and PGI_2 systems has been reported (Cheng *et al*. 2002, Martorell *et al*. 2008) and joint increases in PGI_2 and TXA_2 synthesis have been shown in pathological conditions (FitzGerald 1991, Caughey *et al*. 2001). Therefore, we measured the production of PGI_2 in the presence of furegrelate in mesenteric arteries from both control and orchidectomized rats. First, we found that orchidectomy did not modify either basal or EFS-induced PGI_2 release, in contrast to the increased PGI_2 formation observed in aorta from comparable animals (Martorell *et al*. 2008); these results are in line with reports showing smooth muscle (Wang *et al*. 1993; Ferrer *et al*. 2004) and/or neuronal (Snitsarev *et al*. 2005) cells as cellular sources of PGI_2. Concerning the effect of endogenous TXA_2 on PGI_2 production, we observed that the inhibition of the endogenous TXA_2 synthesis did not modify the release of PGI_2 in arteries from control rats, although it did increase PGI_2 release in arteries from orchidectomized rats. This result reinforced the suggestion that the possible increase in the release of vasodilator substances that counterbalance the vasoconstrictor effect of TXA_2, although the involvement of substances other than PGI_2 can not be ruled out.

In summary, this study demonstrates that non-endothelial TXA_2 release is increased in mesenteric arteries from orchidectomized rats. The effect of endogenous TXA_2 on the EFS-induced response is also regulated in a gonad-dependent way, suggesting that male sex hormones modulate different simultaneous cell signalling pathways. In arteries from control rats, inhibition of TXA_2 formation decreases the EFS-induced response by increasing neuronal NO release, but in arteries from orchidectomized rats, the EFS-induced response is unaltered after the inhibition of TXA_2 formation, by increasing PGI_2 release.

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4

Testosterone and β-estradiol prevent inward remodeling of rat small mesenteric arteries.

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Submitted

ABSTRACT

Increasing evidence shows that sex hormones exert a protective effect on the vasculature, especially in the regulation of the active vasomotor responses. However, whether sex hormones affect vascular remodeling is currently unclear. In the present study, we tested the hypothesis that testosterone in males and β-estradiol in females prevents inward remodeling, possibly through inhibition of the cross-linking enzyme type 2 transglutaminase (TG2). Small mesenteric arteries were isolated from male and female Wistar rats. Using a wire myograph setup, we found a dose dependent relaxation to testosterone and β-estradiol, which was inhibited by the competitive nitric oxide synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME). This confirmed that these hormones were able to induce nitric oxide release. When arteries were cannulated, pressurized, and kept in organ culture with endothelin-1 for three days we observed inward remodeling. This was significantly inhibited by testosterone in males, and by β-estradiol in females. Inward remodeling was also reduced by an inhibitor of TG2, both in males and females. In arteries from female rats, endothelin-1 increased TG2 activity, and this effect was prevented by β-estradiol. We conclude that testosterone and β-estradiol prevent constriction-induced inward remodeling. In females, this effect could be mediated by the inactivation of TG2.

INTRODUCTION

The role of sex hormones has become an important theme in vascular biology during the last decades, as accumulating evidence suggests that both male and female sex hormones may play protective roles on the vasculature [1]. Many studies have demonstrated a beneficial effect of endogenous sex hormones on vascular tone regulation, showing for example, that these hormones increase NO release and/or bioavailability, decrease the oxidative stress, and reduce the synthesis of vasoconstrictor prostanoids [2-5]. Studies regarding the role of sex hormones in vascular remodeling have been mainly focused on growth factors and signal cascades related to smooth muscle cell proliferation [6-8]. However, little is known about the effect of sex hormones on small artery remodeling, which does not necessarily involve cellular proliferation.Small arteries constitute the main source of resistance to blood flow in the vascular system [9]. Regulation of vascular diameter involves adaptation of both vascular tone and structure [10]. Changes in vascular structure include inward remodeling, which is characterized by a decrease in the outer and lumen diameter, and an increase in the media/lumen ratio [11]. The underlying mechanisms of remodeling are still unclear, but it could be the result of a reorganization of the extracellular matrix, with either a repositioning of the cells or a balanced process of apoptosis and proliferation. Inward remodeling has been described in hypertension, in response to low blood flow, and after chronic vasoconstriction [12-14]. It is one of the mechanisms responsible for the increased vascular resistance in hypertension and has a prognostic value for cardiovascular disease [15, 16].In previous work from this group, it has been reported that type 2 transglutaminase (TG2) plays a role in the vascular remodeling associated to chronic vasoconstriction in vitro [17], hypertension [18], and reduced blood flow [19]. The expression of TG2 occurs in several cell types, including the endothelium and smooth muscle cells [20]. TG2 is located both in- and outside of the cell [21] and it has been suggested that its release depends on its activation [22]. An important function of TG2 is the cross-linking of a glutamine residue of a protein/peptide substrate to the primary amino group of a lysine residue [23]. Thus, a large variety of proteins can provide a substrate for the enzyme. The physiological role of TG2 is still being discussed, although its involvement on cell adhesion, wound healing, apoptosis, and matrix reorganization is currently accepted [21]. The activity of TG2 is dependent

on calcium, and can be inhibited by nitric oxide (NO) and GTP [24, 25].

Hence, the objective of the present study was to investigate the effect of testosterone and β-estradiol on constriction-induced remodeling in mesenteric arteries from male and female rats respectively. In addition, we studied the possible role of TG2 in this process.

MATERIAL AND METHODS

Rats and vessel isolation

Small mesenteric arteries from 4 months-old male and female Wistar rats were used. Rats were anesthetized by isoflurane inhalation and sacrificed by decapitation. Artery segments from second and third branches of the mesenteric artery were carefully dissected out and immediately placed in cold MOPS buffer. Arteries were cut in equal-sized pieces where one segment was randomly assigned as control and other segments subjected to various interventions. All experiments were approved by the Committee for Animal Experiments of the Academic Medical Centre Amsterdam (permission 102242).

Wire myograph

Dose-response relationships to sex hormones were recorded using a Mulvany wire myograph (Danish Myo Technology). After dissection vessels were placed in the organ bath containing physiological saline solution (PSS) continuously gassed with 5% CO2/95% O2 air at pH 7.4 and 37ºC. Arteries were mounted on two 40-µm wires and stretched up to its optimal position (normal distension, 0.9 L100), which was estimated according to the Laplace relationship (pressure = tension/radius) that gives an estimated diameter at 100 mmHg [26]. Segments were exposed to PSS containing 120 mmol/l KCl and 10µmol/l norepinephrine to assess its maximum contractility. After a washout period, the integrity of the endothelium was assessed by adding 10µmol/l methacholine to segments precontracted with 10 µmol/l norepinephrine. Then, concentration response curves to β-estradiol (0.1nmol/l -10µmol/l) in females, or testosterone (0.1nmol-10µmol/l) in males were recorded on segments precontracted with 10µmol/l norepinephrine. To determine the contribution of nitric oxide (NO) in this response the competitive NO synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 100µmol/l) was added to the bath 30 minutes prior to the concentration response curves.

Pressure myograph and remodeling

Vessels were also studied using a pressure myograph, where segments were tied to glass cannulas on both ends and pressurized using an electro-pneumatic converter (model T5200, Fairchild). The organ bath was mounted on top of a microscope and

equipped with a digital camera that was connected to a computer. The internal diameter of the vessels was measured using MatLab software (Math Works, USA) and recorded continuously. The temperature of the setup was kept at 37ºC. Vessels were kept in Leibovitz medium containing 1% of a mix of antibiotic-antimycotic solution. The perfusate but not the superfusate was supplemented with 10% heat-inactivated Fetal Calf Serum (HI-FCS). FCS may contain around 20 pg/ml estrogen [27], which corresponds to a final concentration of ~ 7 pmol/l. After checking for leaks, a passive pressure-diameter curve (10-120mmHg) was performed in the presence of papaverine (0.1mmol/l) to rule out the influence of vasomotor tone. After washing out papaverine, the superfusate was supplemented with different compounds depending on the experimental group. Three different groups were used in each gender. For males, the groups consisted of 10nmol/l endothelin-1, 10nmol/l endothelin-1 plus 10nmol/l testosterone, and 10nmol/l endothelin-1 plus 10µmol/l L682.777. For females, the groups consisted of 10nmol/l endothelin-1, 10nmol/l endothelin-1 plus 10nmol/l β-estradiol and 10nmol/l endothelin-1 plus 10µ mol/l L682.777. Arteries were maintained during three days in these conditions at 80 mmHg, with the medium refreshed daily. At the end of the culture period, a second passive pressure-diameter curve (10-120mmHg) was recorded for each artery after full dilation with 0.1 mmol/l papaverine.

Determination of TG2 activity

TG2 activity was visualized using the pseudo-substrate cadaverine linked to FITC (fluorescein isothiocyanate). Arteries were mounted in a pressure myograph, using Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% HI-FCS. In all groups FITC-cadaverine (100µmol/l) was added to the superfusate which was also supplemented with different compounds depending on the group. Three different groups were used in each gender: control, 10nmol/l endothelin-1, and 10nmol/l endothelin-1 plus 10nmol/l testosterone in males; and control, 10nmol/l endothelin-1 and 10nmol/l endothelin-1 plus 10nmol/l β-estradiol in females. The pressure was set at 80mmHg and the myographs were placed in the dark in an incubator at 37ºC during 24 hours. After washing with PBS during 5 minutes and fixation with formaldehyde for 10 minutes, vessels were mounted on glass slides using Vectashield/DAPI and imaged on a confocal microscope (Leica TCS SP2). Laser and photomultiplier settings were unchanged during the acquisition of images from the different groups in each rat. TG2 activity was quantified using ImageJ software. Data were corrected for vessel size and

depicted in arbitrary units.

Calculations and statistics

From each rat, 3-4 equal sized segments were obtained and studied in simultaneous experiments, being assigned to different experimental groups. Results are expressed as mean ± SEM for the number of animals indicated. T-tests were used to compare data, followed by Bonferroni corrections as appropriate. To compare pressure-diameter relations on day 0 vs. day 3, two-way Repeated-Measures ANOVAs were also performed. Data were considered significant when $p < 0.05$.

Chemicals and solutions

Leibovitz medium, DMEM medium and the antibiotic-antimycotic solution were obtained from GIBCO (Breda, The Netherlands). FCS was obtained from BioWhitaker (Verviers, Belgium). Papaverine, norepinephrine, methacholine, L-NAME and β-estradiol were obtained from Sigma (Sigma-Aldrich, St. Louis, MO) and testosterone was obtained from Fluka (Sigma-Aldrich, St. Louis, MO). Endothelin-1 was obtained from Bachem (Bubendorf, Switzerland), L682.777. was obtained from Zedira, (Darmstadt, Germany). Salts were obtained from Merck (Darmstadt, Germany). FITC-cadaverine was obtained from AnaSpec Inc. (Fremont, CA). Vectachield/DAPI was obtained from Vector Laboratories (Burlingame, CA). MOPS buffer was composed of (in mmol/l): 145 NaCl, 4.7 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 2 CaCl2, 3 3-(N-morpholino) propanesulfonic acid, 5 glucose, and 2 pyruvate, pH 7.4. PSS buffer was composed of (in mmol/l) 119 NaCl, 25 NaHCO3, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4, 2.5 CaCl2, 0.027 EDTA and 5.5 glucose.

RESULTS

Vascular reactivity

The acute effect of testosterone and β-estradiol on vascular function was tested in a wire myograph setup. Mesenteric arteries were pre-contracted with norepinephrine. Then, male vessels were exposed to increasing doses of testosterone. This induced a dose dependent relaxation (Figure 1). Segments from female rats were exposed to increasing doses of β-estradiol, which also induced dose dependent relaxation. The relaxation induced by both sex hormones was attenuated in the presence of the competitive NO synthase inhibitor, L-NAME (Figure 1). This shows that both hormones induce relaxation through release of NO.

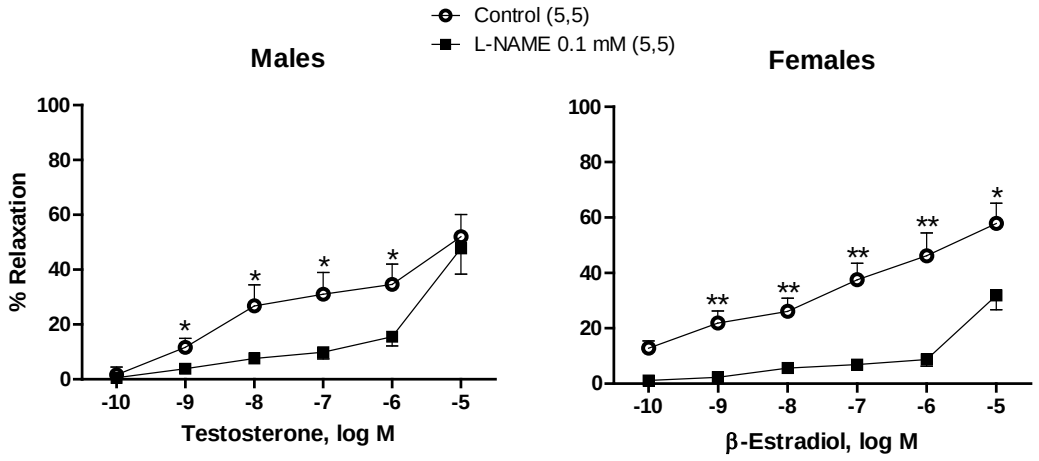

Figure 1. Testosterone and β-estradiol induced a dose-dependent relaxation in small mesenteric segments from male (A) or female (B) rats, respectively. Incubation with L-NAME partially prevented this relaxation in both groups. Results (mean ± SEM) are expressed as the percentage of inhibition of contraction induced by 10µmol/L norepinephrine. Number of animals is indicated in parenthesis.

Vascular remodeling

To study the effect of physiological concentrations of sex hormones on inward remodeling associated with chronic vasoconstriction, we used an organ culture approach. Small mesenteric arteries were cannulated and exposed to endothelin-1 for 3 days. Arteries from both genders showed inward remodeling after the culture period (Figures 2 and 3). Remodeling was defined as a difference in the passive diameter between the first and the second measurement at a given pressure in each segment. When testosterone was also added to the culture medium of male arteries (Figure 2a),

or β-estradiol to the culture medium of female arteries (Figure 2b), remodeling was reduced. This showed that both sex hormones partially prevent constriction-induced vascular remodeling. Remodeling was also partially prevented in both genders by incubation with the TG2 inhibitor L682.777 (Figures 3a and 3b), indicating that TG2 is involved in the remodeling process.

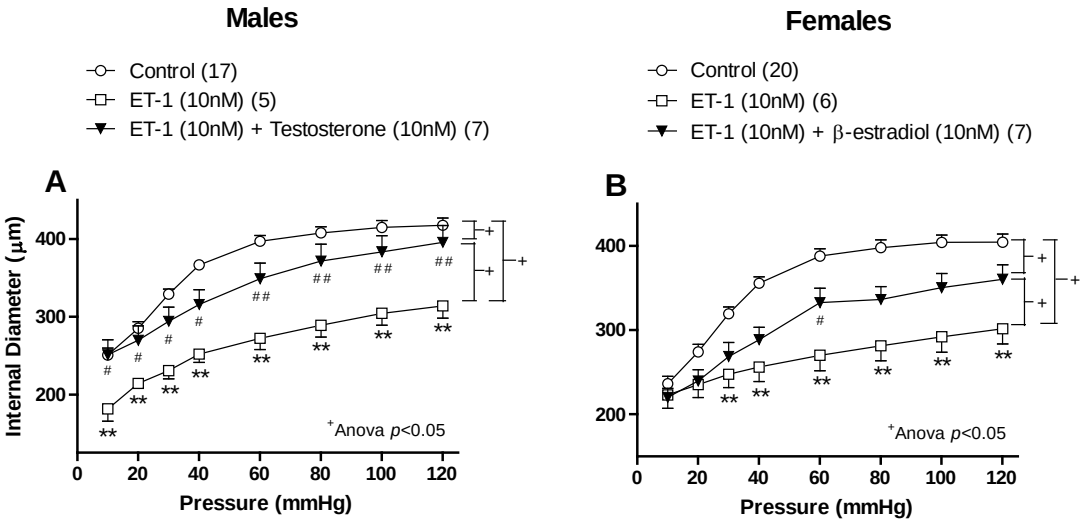

Figure 2. Passive pressure-diameter curves of cannulated small mesenteric arteries were recorded to measure remodeling. After the culture period, arteries from both genders incubated with endothelin-1 (ET-1) showed inward remodeling. (A) When male arteries were also incubated with testosterone, remodeling was attenuated. (B) Remodeling was also attenuated by β-estradiol in female arteries. Results are expressed as mean ± SEM. Number of animals is indicated in parenthesis. RM Two-way anova: +p<0.05. T-test: control vs ET-1, **p<0.01; ET-1+Testosterone (A) or ET-1+β-estradiol (B) vs. ET-1, #p<0.05, ##p<0.01.

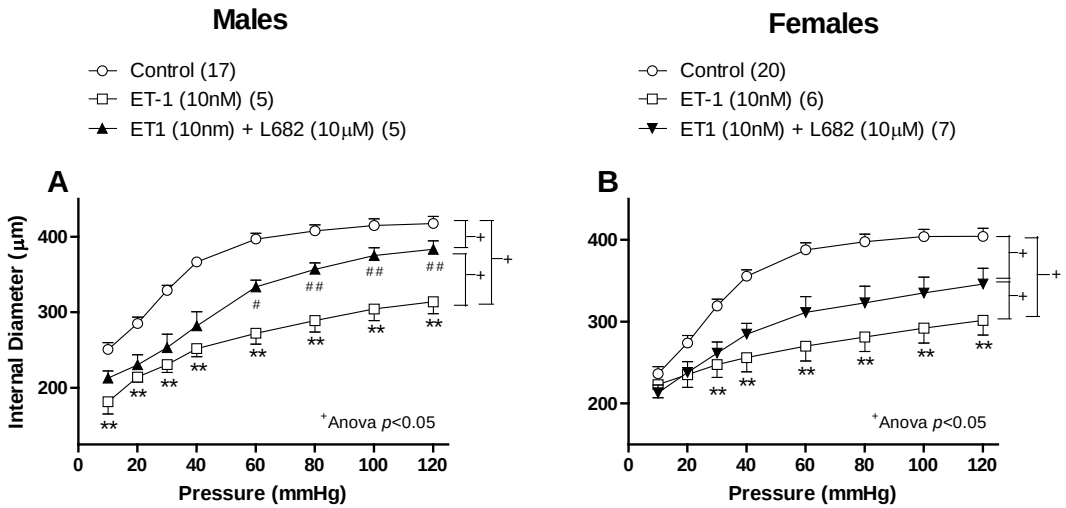

Figure 3. Inward remodeling induced by endothelin-1 (ET-1) was prevented by the TG2 inhibitor L682.777 (L682) in both genders (A, B). Results are expressed as mean ± SEM. Number of animals is indicated in parenthesis. RM Two-way ANOVA: $^{+}p<0.05$. T-test: control vs ET-1, $^{**}p<0.01$; ET-1+L682 vs. ET-1, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

During the three days of incubation, the constriction induced by endothelin-1 was maintained, and this was not modified by coincubation with testosterone, β-estradiol or L682.777 (Figure 4).

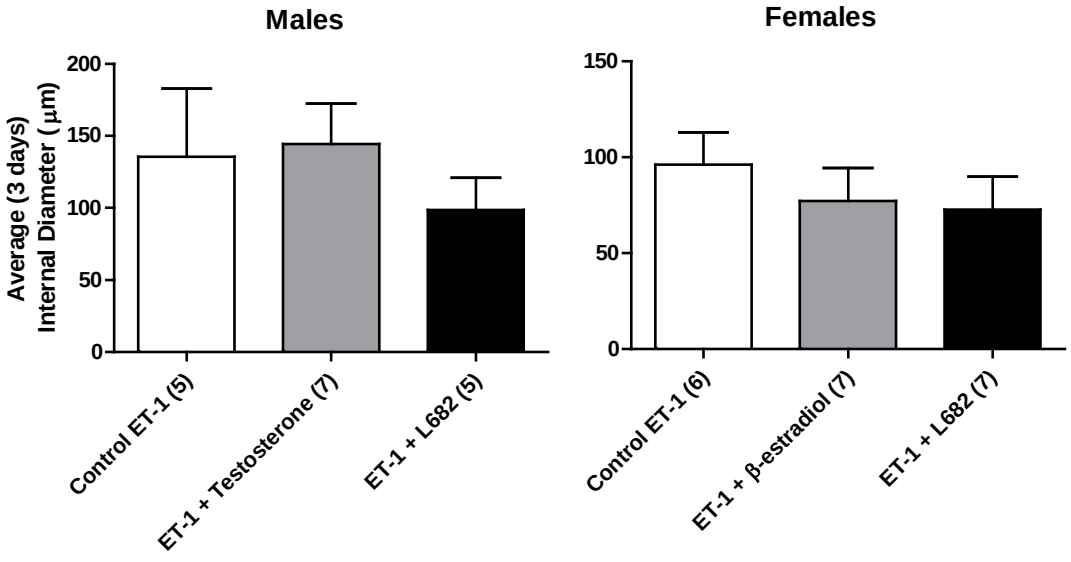

Figure 4. Contraction induced by endothelin-1 expressed as the average internal diameter, was not significantly modified by any intervention during the culture period in small mesenteric segments from male or female rats. Results are expressed as mean ± SEM. Number of animals is indicated in parenthesis.

Transglutaminase activity

Since we found that ET-1-induced remodeling depends on both TG2 and sex hormones, we studied the relationship between the sex hormones and TG2 activity. The activity of TG2 in the vessel wall is reflected by the incorporation of fluorescent cadaverine (FITC-cadaverine), a substrate for TG2 (Figure 5). In males, endothelin-1 did not significantly increase TG2 activity. Testosterone also did not modify TG2 activity. In females, ET-1 induced a significant increase in TG2 activity. This increase was completely abolished in vessels incubated with β-estradiol.

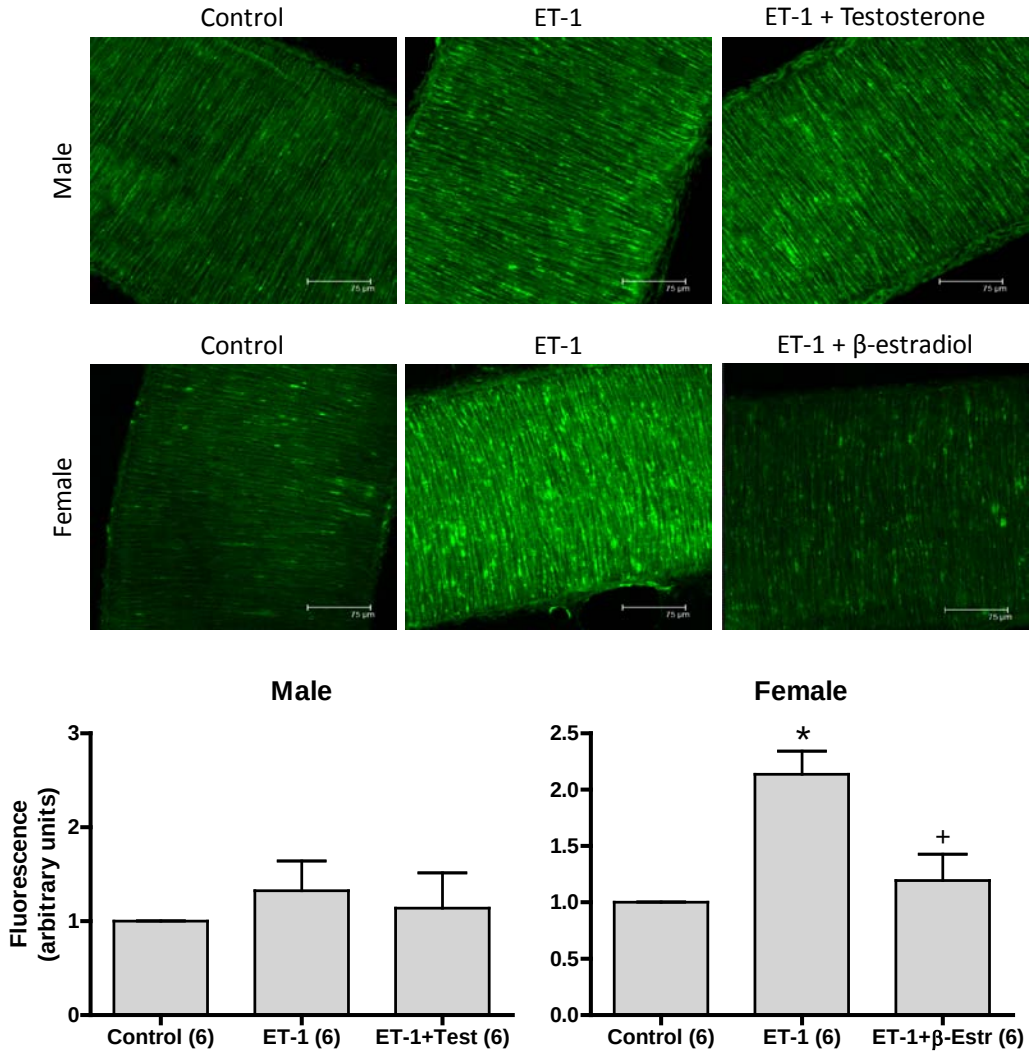

Figure 5. Confocal images of small mesenteric segments from male and female rats. TG2 activity is shown in green, detected by the incorporation of the pseudo substrate FITC-cadaverine. TG2 activation induced by ET-1 was prevented by β-estradiol in segments from female rats. In segments from male rats, TG2 was not activated by ET-1, and this was not modified by testosterone. Data are expressed as mean ± SEM. Number of animals is indicated in parenthesis. *p<0.05 Control vs ET-1. +p<0.05, Control vs ET-1+ β-estradiol.

DISCUSSION

The present study aimed to investigate the effect of sex hormones on vascular tone and structure. Clinical and epidemiological studies have shown an inverse correlation between serum sex hormones levels and cardiovascular disease in both genders [28-30]. Serum levels of sex hormones decrease with age, and evidence suggests that they play an important role in the regulation of vascular function [1, 31-33]. Thus, low serum levels are independently related to cardiovascular disease in both genders [34-36]. The protective effect of sex hormones can be attributed to several of their actions, such as modulation of the lipid profile, vascular tone and vascular structure [2, 31, 33]. Even so, studies concerning vascular structure and sex hormones have focused mainly on the hypertrophic remodeling, and there are few data concerning the effect of sex hormones on inward remodeling [37-39]. Here we tested whether testosterone and β-estradiol (the main male and female sex hormones, respectively) could prevent vascular inward remodeling induced by three days of maintained constriction with endothelin-1 under pressurized conditions. We observed that in both genders, corresponding hormones were able to inhibit vascular remodeling.

Previous work showed that an active, persistent constriction is necessary to induce eutrophic inward remodelling. Consequently, remodeling could be prevented by vasodilators such as papaverine and verapamil [13]. Considering these data, the reported effect of the hormones could be related to its vasodilator properties. The wire myograph experiments showed that both sex hormones indeed induce a dose-dependent relaxation. This relaxation was inhibited by L-NAME, indicating it is mediated by NO. However, the analysis of the average tone of the pressurized arteries during the three days of organoid culture revealed that constriction was similar during the culture period in all groups. This indicates that the inhibitory effect of sex hormones on inward remodeling was not related to its vasodilator effect.

Vascular remodeling of small arteries induced by reduced blood flow, hypertension, and exposure to vasoconstrictors depends on TG2 [17-19]. Thus, inhibition of the activity of the enzyme prevents vascular remodeling [17, 19, 40], an observation that was confirmed in the present study. Although the way by which TG2 exerts its effect on remodeling is not totally clear, TG2 could play a role in providing mechanical strength to the vessel wall by cross-linking structural proteins within the extracellular matrix. It has

been suggested that acting this way, TG2 could fixate the constricted artery in a more narrowed state [13, 41]. Some proteins have been found to be substrates for TG2 in the extracellular matrix of remodeling-activated smooth muscle cells, such as fibronectin, collagen alpha-1 chain, fibulin-2 and nidogen-1 precursors [42]. Other data from the same publication showed that TG2 activity associated to remodeling is located to the cell membrane, suggesting that translocation of the enzyme is necessary to induce remodeling. Once activated and located in the extracellular cell surface, TG2 could cross-link its extracellular substrates.

We found that in females, endothelin-1 increased TG2 activity. This increase in TG2 activity was completely inhibited by addition of β-estradiol. This suggests that the inhibitory effect of β-estradiol on remodeling is mediated by modulation of TG2 activity. An attractive hypothesis is that the effect of β-estradiol on TG2 is mediated by NO. It has been reported that NO release induced by sex hormones induces S-nitrosilation of different proteins [43-45]. It has also been reported that TG2 can be inactivated by S-nitrosylation [24, 46]. In fact, TG2 activation and associated remodeling can be inhibited by NO donors [17, 42]. Thus, despite the fact that the level of tone was not significantly affected by β-estradiol during culture, we speculate that β-estradiol inhibits TG2 activity through release of NO.

In males, endothelin-1 did not significantly increase TG2 activity, and testosterone did not modify this. Thus, although testosterone prevented inward remodeling, it does not seem to act through modulation of TG2 activity. Further work is therefore necessary to clarify the inhibitory effects of testosterone on remodeling in males. Conversion of testosterone to β-estradiol trough aromatase, which is present in both smooth muscle and endothelial cells [47] could be speculated upon. However, since neither endothelin-1 nor testosterone affected TG2 activity, a similar pathway in males as in females via the conversion of testosterone to β-estradiol appears unlikely. Taken together, these data show that the mechanism of action of sex hormones is different in each gender. The incidence of cardiovascular disease is lower in women compared to age-matched men but it increases notably in women after menopause [36, 48]. This effect led to the idea that female sex hormones were vascular protective while male sex hormones were deleterious although more recent evidence indicates that also male sex hormones play a preventive role in the vascular function [33]. Nevertheless, the way by which sex hormones from both genders exert this protection is different, and often female sex hormones seem to be more efficient in this respect. Interestingly, regarding the

stiffening of the vessel wall, women exhibit a greater age-related rise in arterial stiffness after menopause compared to aged matched men [38], and this difference seems to be due to the intrinsic gender differences of the arteries, that become evident when the sex steroid secretion is low. Nevertheless, sex hormones also modulate this stiffness [37]. Thus, apart from the differences in the action of the hormones, specific gender properties of the tissue may determine the way of action of sex hormones.

We conclude that physiological concentrations of testosterone in males and β-estradiol in females prevent constriction-induced remodeling. This effect does not seem to be due to the vasoactive action of sex hormones. In females, the beneficial effect of β-estradiol may be due to the inhibition of TG2. These observations could be of relevance for the treatment of human cardiovascular disease through hormone replacement; since the process of small artery inward remodeling that is seen in hypertension [9] may be reversible at its early stages [49]. Studies in humans on large arteries have also shown an inverse relation between serum hormone levels and arterial stiffness, which can be reversed when serum levels are restored [37]. Further studies should therefore address the possibility that the hormone replacement therapy could reverse or at least prevent vascular remodeling.

Author contributions

Lara del Campo contributed to the conception and design of the study, collected analyzed and interpreted the data, and drafted the article. Bilge Guvenc Tuna collected, analyzed and interpreted some data. Mercedes Ferrer contributed to the conception and critical revision of the study. Ed van Bavel contributed to the conception and critical revision of the study. Erik NTP Bakker contributed to the conception and design of the study, collected, analyzed and interpreted the data, drafted the article and contributed to the critical revision of the study.

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5

CONCLUSIONS

CONCLUSIONS

The results presented in this thesis allow to conclude that male sex hormones influence vascular tone and vascular structure.

Regarding the neuronal control of vascular tone the results show that:

- Orchidectomy does not alter NA release induced by EFS, but it does increase the EFS-induced TXA_2 release, suggesting that TXA_2 could be the contractile factor that maintains the EFS-induced vasoconstrictor response in orchidectomized animals.
- Orchidectomy modifies the role of TXA2 in the EFs-induced response:
  - In arteries from control rats, inhibition of TXA_2 synthesis decreases the vasoconstrictor response to EFS, not only because the vasoconstrictor effect of TXA_2 is eliminated, but also because this inhibition increases the release and the response to NO.
  - In arteries from orchidectomized rats, inhibition of TXA_2 synthesis does not alter the response to EFS because the absence of TXA_2 is counterbalanced by a high increase in PGI_2 production, which mediates vasoconstriction.
- Post-synaptic β-adrenoceptor function in smooth muscle cells is not modulated by endogenous male sex hormones, as the response to exogenous NA, the NO donor SNP or clenbuterol is not modified by orchidectomy.
- Endogenous male sex hormones modulate the function of pre-synaptic β-adrenoceptors. In orchidectomized rats, pre-synaptic β-adrenoceptor activation induces an increase in the release of NA and neuronal NO that compensate each other so that the vasoconstrictor response to EFS is not altered.

Regarding vascular structure it was found that:

- Physiological concentrations of testosterone inhibit inward remodeling induced by chronic vasoconstriction in small mesenteric arteries from male rats.
- The preventive effect of testosterone on vascular remodeling may be due to mechanisms different from TG2 inactivation.

CONCLUSIONES

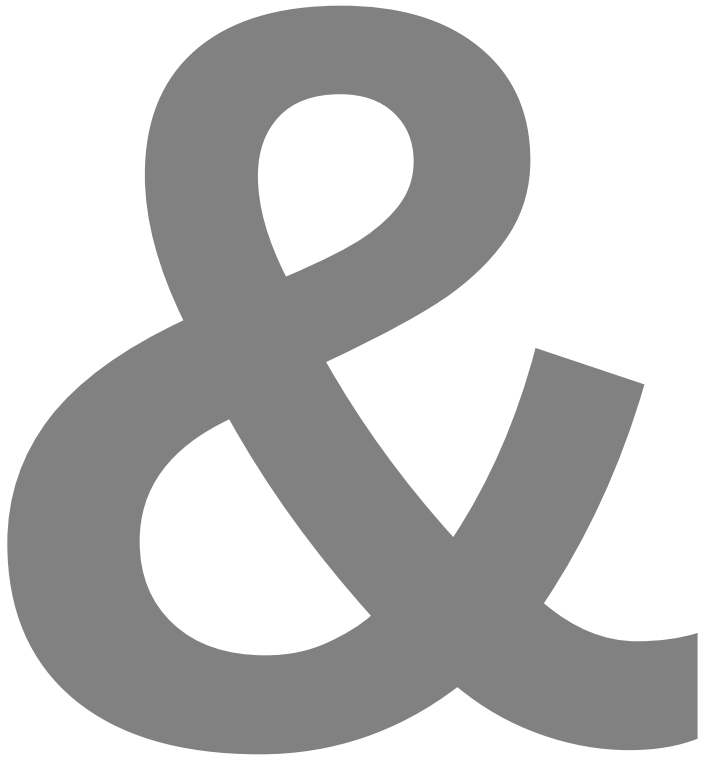
Los resultados de esta tesis permiten afirmar que el control del tono y la estructura vasculares están bajo la influencia de las hormonas sexuales masculinas.

Con respecto al control neuronal del tono vascular los resultados muestran que:

- La orquidectomía no modifica la liberación de NA inducida por EE, aunque si incrementa la liberación de TXA_2, lo cual sugiere que este prostanoide podría compensar la disminución del efecto vasoconstrictor de la NA en animales orquidectomizados manteniendo la respuesta a EE.
- La orquidectomía modifica el papel del TXA_2 en la respuesta contráctil inducida por EE:
    - En arterias de ratas controles, la inhibición de la síntesis de TXA_2 disminuye la respuesta vasoconstrictora a EE, no sólo porque se elimina el efecto vasoconstrictor del TXA_2 sino también porque esta inhibición incrementa la liberación y la respuesta a NO.
    - En arterias de ratas orquidectomizadas, la inhibición de la síntesis de TXA_2 no modifica la respuesta a EE, porque la ausencia de TXA_2 se compensa con un incremento en la liberación de PGI_2 que tiene un efecto vasoconstrictor.
- La función de los receptores β-adrenérgicos postsinápticos no parece estar influenciada por las hormonas sexuales masculinas ya que la respuesta vasomotora a NA exógena, al donante de NO (SNP) o al clenbuterol no se ve modificada por la orquidectomía.
- La orquidectomía modula la función de los receptores β-adrenérgicos presinápticos. En animales orquidectomizados, la activación de los receptores β-adrenérgicos induce un incremento en la liberación de NA y NO neuronal cuyos efectos vasomotores se compensan, haciendo que finalmente, la respuesta vasomotora a la EE no se modifique.

Con respecto al control de la estructura vascular, los resultados muestran que:

- El remodelado hacia el interior inducido por la vasoconstricción mantenida durante 3 días disminuye en presencia de concentraciones fisiológicas de testosterona en arterias de resistencia de ratas macho.
- El efecto preventivo de la testosterona en machos parece no deberse a la inhibición de la enzima TG2.



APPENDIXES

LIST OF ABBREVIATIONS

^{3}H	Tritium
AA	Arachidonic acid
Ach	Acetylcholine
ADRF	Adipose derived relaxing factor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
Clen	Clenbuterol
COX	Cyclooxigenase
CRLR	Calcitonin receptor-like receptor
DAF	Diaminofluorescein
DAG	Diacylglycerol
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular cell matrix
EDHF	Endothelium derived hyperpolarizing factor
EFS	Electrical Field Stimulation
eNOS	Endotelial nitric oxide synthase
ET-1	Endothelin-1
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GTP	Guanosine triphosphate
HI	Heat-inactivated
iNOS	Inducible nitric oxide synthase
IP_3	Inositol triphosphate
K^+	Potassium
KHS	Krebs-Henseleit solution
L-NAME	N_ω-Nitro-L-arginine methyl ester hydrochloride
L-NMMA	L-NG-monomethyl arginine
NA	Noradrenaline
nNOS	Neuronal nitric oxide synthase
NO	Nitric Oxide
NPY	Neuropeptide Y
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrite
PGD	Prostaglandin D
PGE_2	Prostaglandin E_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PIP_2	Phosphatidyl-inositol bisphosphate
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
Prop	Propanolol
PSS	Physiological saline solution
RAMP1	Receptor-affinity-modifying protein 1
ROS	Reactive Oxigen Species
SEM	Standard error of the media
SNP	Sodium nitroprusside
TG2	Transglutaminase 2
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
VIP	Vasoactive intestinal polypeptide

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